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In the previous reporting periods, we showed that cadherin binding is necessary to recruit p120 to cell junctions and that p120 is important to the generation of strong cell-cell adhesion. We have extended these observations to show that p120 acts at the cell surface to control cadherin turnover, thereby directly regulating cadherin levels. p120 knockdown by siRNA expression results in dose-dependant elimination of E-, P-, N-, and VE-cadherins, and complete loss of cell-cell adhesion. p120 family members ARVCF and δ-catenin are functionally redundant. The data reveal the core function of p120 in cadherin complexes, and strongly predict a dose-dependant loss of E-cadherin in tumors that partially or completely downregulate p120. These data suggest a crucial and largely unrecognized tumor suppressor role for p120, which will guide further studies aimed at determining whether p120 loss contributes to tumor progression in the breast.

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INTRODUCTION

This work was undertaken to elucidate the role of p120ctn in E-cadherin-mediated cell-cell adhesion. E-cadherin is the epithelial-specific member of a large class of transmembrane, cell-cell adhesion molecules. Loss of E-cadherin is widely regarded as a transitional event leading to metastasis in the tumor progression of epithelial tissues. As 90% of all cancers are epithelial in origin, understanding E-cadherin-mediated cell-cell adhesion and E-cadherin loss is of considerable importance. p120ctn is one of several proteins that bind E-caderin's cytoplasmic domain, and evidence suggests that it may be a key regulator of E-cadherin function. We have mutated the cytoplasmic domain of E-cadherin to map the specific binding domain of p120 and to generate mutant E-cadherin molecules which are unable to bind p120. Expression of these mutants indicates that p120 binding to E-cadherin is necessary for the tight adhesion typical of epithelial cells. Furthermore, using p120-specific siRNA we show that p120 is critical for the stability of the E-cadherin protein. Surprisingly, p120 knockdown results in loss of E-cadherin, and near complete loss of cell-cell adhesion. We conclude that p120 regulates cadherin turnover, and that p120 loss in tumors is likely to contribute to tumor progression.

STATEMENT OF WORK

This "STATEMENT OF WORK" has been provided as a reference for the susequent "BODY".

Task 1: To generate the MDA231 model system for uncoupling E-cadherin and p120 function (months 1-12).

A. Subclone E-cadherin AAA mutants from yeast pGad vector to pLKpac1 expression vector.
B. Transfect E-cadherin mutants into MDA231 cells and establish stable cell lines expressing these mutants.
C. Screen cell lines for E-cadherin expression by immunofluorescence, and western blotting.

Task 2: To characterize stably transfected cell lines and identify minimal E-cadherin JMD mutants that effectively uncouple E-cadherin from p120 (months 6-18).

A. Characterize binding of wild-type and mutant E-cadherin to p120 and α- and β-catenin by co-immunoprecipitation.
B. Determine final mapping of p120 binding site on E-cadherin's JMD.
C. Perform subcellular fractionation of transfected cells to address p120/E-cadherin stiochiometry.
D. Phosphatase-treat p120 immunoprecipitates and blot for p-tyr, ser and thr to assess phosphorylation state of p120 when bound to or uncoupled from E-cadherin.

Task 3: To determine the impact of uncoupling p120 and E-cadherin on adhesion, motility, and invasion by performing functional assays *in vitro*. (months 12-36).

A. Perform aggregation assays using established protocols for cadherin function.
B. Examine motility using an in vitro wound healing assay.
C. Perform invasion assays in soft agar.

Task 4: To analyze the effects of uncoupling p120 and E-cadherin on metastasis utilizing a mouse model for metastasis of breast cancer to bone (months 24-36)

A. Inject 24 female Balb/c-nu/nu (nude) mice with MDA231 cells transfected with vector alone, wild-type E-cadherin, or two different mutants of E-cadherin which do not bind p120 (6 mice in each group).
B. Assess tumor size (radiographically) and number in animals.
C. Test ability of tumors to metastasis to bone using histological examination.
D. Measure growth rate and tumorigenicity of cells by injection into the mammary fat pad of nude mice.

BODY

We have completed Tasks 1, 2, and 3 and most of these results are published in the first reference in the appendix (Thoreson et. al., 2000). For the remainder of this discussion, I will refer to figures in the three papers included in the appendix.

For task 1, stable cell clones were generated from cells transfected with p120-uncoupled mutants of E-cadherin (Fig.s 4, 6, and 9). We demonstrate that unlike the other catenins binding E-cadherin, p120 is stable when uncoupled from E-cadherin (Fig. 1B). Futhermore, we show that E-cadherin is required for p120 localization at the membrane in these cells, and without E-cadherin, p120 localizes to the cytoplasm (Fig. 1A and 1B, Thoreson et. al., 2000).

For Task 2, we characterized the cell lines obtained in Task 1 by performing co-immunoprecipitations of p120 with the E-cadherin mutants. We found that p120 does not bind to these E-cadherin mutants and that the binding of α-catenin and β-catenin to these mutant is no different from their binding to wild type E-cadherin (Fig. 5). Subcellular fractionation suggests that over 90% of p120 associates with E-cadherin, demonstrating a high stoichiometric interaction (Fig. 7). Phosphatase treatment of cells confirmed that p120 is phosphorylated when bound to cadherin, but not phosphorylated while cytoplasmic (Fig. 8).

Task 3 was completed by performing several assays for cadherin function. Cells containing mutant E-cadherin had loose junctions and were unable to form tight colonies on tissue culture plates (Fig. 9B). Actin staining revealed disorganized actin filaments in cells containing mutant E-cadherin, and circumferential actin cables were absent from mutant cells, even though cadherins were able to concentrate at junctions Fig. 9A). Aggregation assays revealed a selective inability of the mutant E-cadherin to promote strong adhesion (Fig. 9B). These observations suggest a defect in cadherin clustering due to lack of p120 binding.

Due to the instability of E-cadherin expression in generated cell lines, we were unable to complete Task 4 as described within the "STATEMENT OF WORK". However, consistent with the goals of this project, we were able to take a different approach to show that p120 is required for stabilization of E-cadherin at the cell surface (see appendix: Davis et.al., Journal of Cell Biology, In Press, 2003). We knocked down p120 in A431 cells using siRNA methods (Fig. 1a) and showed that E-cadherin levels decreased dramatically Fig. 1c), resulting in loss of cell-cell adhesion (Fig. 1b). p120 apparently controls the levels of all cadherins, not just E-cadherin (Fig. 2). Moreover, p120 family members are redundant for this function (Fig. 3). This work reveals for the first time a core function of p120 in the cadherin complex. P120 essentially regulates cadherin turnover at the cell surface, thereby controlling cell-cell adhesion (Fig.'s 5, 6, and 7). Recent evidence indicates frequent loss of p120 in various tumor types, including breast (see appendix, Thoreson et. al., 2002). We suggest that cadherin – loss in some tumors may be preceded by, and indeed caused by loss of p120. Overall, the data indicate that p120 regulates cadherin turnover, and in some circumstance may act as a tumor suppressor (Davis et. al., In Press; Thoreson and Reynolds, 2002).

KEY RESEARCH ACCOMPLISHMENTS

- Showed that p120ctn localizes to the cytoplasm in cadherin deficient cells, where it is stable but unable to be phosphorylated, and demonstrated that cadherin expression is necessary and sufficient for p120 localization to cell junctions.

- Uncoupled p120 from E-cadherin through mutations in the juxtamembrane domain of E-cadherin and showed that p120ctn is necessary for strong adhesion and possibly cadherin clustering.

- Knocked down p120ctn with siRNA, and showed that a core function for p120ctn in cadherin complexes is to regulate cadherin turnover.
- Provided evidence that p120 is a tumor suppressor.

REPORTABLE OUTCOMES

- Publications:
  - Ireton RC, Davis MA, van Hengel J, Mariner DJ, Barnes K, Thoreson MA, Anastasiadis PZ, Matrisian L, Bundy LM, Sealy L, Gilbert B, van Roy, F, and Reynolds AB. (2002) A novel role for p120 catenin in E-cadherin function. Journal of Cell Biology, 159: 465-476.
  - Thoreson MA and Reynolds AB. Altered Expression of the Catenin p120 in Human Cancer: Implications for Tumor Progression. *Differentiation*. 2002 Dec; 70(9-10):583-9
  - Davis MA, Ireton RC, and Reynolds AB. A core function for p120-catenin in cadherin turnover. (2002) Journal of Cell Biology, 163: 525-534.

- Molly Thoreson Recieved her Ph.D. from the Department of Cancer Biology at Vanderbilt University in May 2002. She accepted a short-term post-doctoral position in the lab of Al Reynolds, and was subsequently hired by Amgen, Inc. as an Oncology Medical Liason.

CONCLUSIONS

Through this research, we have taken enormous strides in understanding p120ctn's role in regulating cadherin function. Specifically, we show that p120ctn is necessary for strong adhesion of the cadherin complex and show that E-cadherin is rapidly degraded in the absence of p120. The data reveal for the first time, a core function of p120 in regulating cadherin turnover. Moreover, based on these data, and evidence that p120 is frequently turned off in tumors, it appears that p120 may have an essential role as a tumor suppressor. We have generated conditional knockout mice, and seek to determine the consequences of p120 knockout in the breast, and whether it indeed functions as a tumor suppressor.

REFERENCES

Ireton RC, Davis MA, van Hengel J, Mariner DJ, Barnes K, Thoreson MA, Anastasiadis PZ, Matrisian L, Bundy LM, Sealy L, Gilbert B, van Roy, F, and Reynolds AB. (2002) A novel role for p120 catenin in E-cadherin function. Journal of Cell Biology, 159: 465-476.

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BIBLIOGRAPHY

See "Reportable Outcomes" and "Appendices".

APPENDICES

Three publications, listed above in "reportable outcomes" and in "References").

JCB Article

A novel role for p120 catenin in E-cadherin function

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Indirect evidence suggests that p120-catenin (p120) can both positively and negatively affect cadherin adhesiveness. Here we show that the p120 gene is mutated in SW48 cells, and that the cadherin adhesion system is impaired as a direct consequence of p120 insufficiency. Restoring normal levels of p120 caused a striking reversion from poorly differentiated to cobblestone-like epithelial morphology, indicating a crucial role for p120 in reactivation of E-cadherin function. The rescue efficiency was enhanced by increased levels of p120, and reduced by the presence of the phosphorylation domain, a region previously postulated to confer negative regulation. Surprisingly, the rescue was associated with substantially increased levels of E-cadherin. E-cadherin mRNA levels were unaffected by p120 expression, but E-cadherin half-life was more than doubled. Direct p120–E-cadherin interaction was crucial, as p120 deletion analysis revealed a perfect correlation between E-cadherin binding and rescue of epithelial morphology. Interestingly, the epithelial morphology could also be rescued by forced expression of either WT E-cadherin or a p120-uncoupled mutant. Thus, the effects of uncoupling p120 from E-cadherin can be at least partially overcome by artificially maintaining high levels of cadherin expression. These data reveal a cooperative interaction between p120 and E-cadherin and a novel role for p120 that is likely indispensable in normal cells.

Introduction

p120-catenin (p120)* is a member of the Armadillo (ARM)/β-catenin gene family (Reynolds et al., 1992; Peifer et al., 1994), and the prototypical member of the p120 subfamily (for review see Anastasiadis and Reynolds, 2000). Originally described as a substrate for the Src oncoprotein (Reynolds et al., 1989, 1992) and various receptor tyrosine kinases (Downing and Reynolds, 1991; Kanner et al., 1991), p120 interacts with the cytoplasmic domain of classical cadherins (Reynolds, 1994; Shibamoto et al., 1995; Staddon et al., 1995). Cadherins are both necessary and sufficient for the targeting of p120 to cell–cell junctions, indicating that cadherins are the only cellular proteins capable of recruiting p120 to membranes (Thoreson et al., 2000). The p120 binding site in the juxtamembrane domain (JMD) of cadherins is the most highly conserved region among members of the cadherin superfamily (Nollet et al., 2000), suggesting a general and indispensable role for the cadherin-p120 interaction.

Cadherins comprise a superfamily of transmembrane cell–cell adhesion receptors involved in many aspects of development, morphogenesis, and tumor malignancy (for reviews see Nollet et al., 1999; Takeichi, 1995; Yap, 1998). Extracellular domains of identical cadherins interact in a homophilic, Ca^{2+}-dependent fashion to form adherens junctions between adjacent cells. The cytoplasmic domains interact with the catenins, which physically connect and/or regulate the interaction of the cadherin complex with the actin cytoskeleton. β-Catenin and p120 bind through their Armadillo repeat domains to the catenin binding domain and JMD of cadherins, respectively (Aberle et al., 1994; Hulsken et al., 1994; Funayama et al., 1995; Jou et al., 1995; Navarro et al., 1998; Yap et al., 1998). Compared with β-catenin, p120 is rather loosely associated with the cadherin complex (Thoreson et al., 2000), a property that may be important for its mechanism of action (Anastasiadis and Reynolds, 2001).

The role of p120 is controversial. Evidence suggests that p120 can both positively and negatively regulate cadherin activity, probably depending on signaling events that have yet to be clearly identified. JMD-deleted cadherins have been

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*Abbreviations used in this paper: ARM, armadillo; IRES, internal ribosomal entry site; JMD, juxtamembrane domain; neo, neomycin resistance gene; p120, p120-catenin; PS, presenilin.

Key words: p120ctn; p120; cadherin; catenin; SW48

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used to assay cadherin function in the absence of p120 binding. These studies suggest roles for the cadherin JMD, and by implication p120, in developmental events (Kintner, 1992; Horikawa and Takeichi, 2001), neuronal outgrowth (Riehl et al., 1996; Lilien et al., 1999), exclusion of one cadherin from junctions by another (Navarro et al., 1998), suppression of cell motility (Chen et al., 1997), cadherin clustering (Yap et al., 1998), and cadherin adhesiveness (Yap et al., 1998; Thoreson et al., 2000). Minimal p120-uncoupling mutations (e.g., triple alanine substitutions) have been used to minimize the risk of affecting non-p120 interactions (Thoreson et al., 2000). Nonetheless, novel JMD binding partners have been identified which are uncoupled by the same mutations that eliminate p120 binding. Both Hakai (Fujita et al., 2002) and presenilin (PS)-1 associate with the JMD (Baki et al., 2001; Marambaud et al., 2002), and have been implicated in mechanisms that down-regulate cadherin levels. The latter observations complicate the interpretation of p120's contribution to the results of JMD-deletion experiments.

p120 function has been more directly addressed in other systems. For example, in Colo205 cells, E-cadherin function is severely impaired despite the cells having normal levels of E-cadherin and catenins. Adhesion can be restored by p120 mutants lacking most of the NH_2-terminal region, but not by full-length p120 (Aono et al., 1999). This result implies an aberrant signaling pathway in Colo205 cells that acts constitutively through an NH_2-terminal region of p120 to block E-cadherin function. Although aspects of these data have been questioned recently (Horikawa and Takeichi, 2001), the ability of NH_2-terminally truncated p120 to restore adhesiveness in these cells is clear. Thus, full-length p120 appears to negatively regulate adhesion in Colo205 cells.

p120 contains a 350-aa NH_2-terminal region, followed by the ARM domain (i.e., ten ARM repeats), and a short COOH terminus. The NH_2-terminal region immediately adjacent to the ARM domain encompasses the 100-aa phosphorylation domain which contains the majority of the p120 tyrosine phosphorylation sites (Mariner et al., 2001) and is probably an important regulator of p120 function. Most cells simultaneously express multiple p120 isoforms derived from alternative splicing. Splicing in the NH_2-terminal region gives rise to four alternative start codons (Keirsebilck et al., 1998). Isoforms using start codons 1 (p120ctn1) and 3 (p120ctn3) are observed most frequently (Mo and Reynolds, 1996; Keirsebilck et al., 1998) and retain both the phosphorylation domain and the ARM domain. The fourth start codon gives rise to isoform 4 (p120ctn4), which retains the ARM domain but eliminates the NH_2-terminal region, along with its phosphorylation domain. In contrast to other isoforms, p120ctn4 mRNA has been detected, but is rarely expressed as a protein. It is used in our study as a p120 mutant lacking the NH_2 terminus.

E-cadherin is well established as a tumor and metastasis suppressor. Expression of E-cadherin is frequently down-regulated or extinguished in malignancy, an event strongly correlated with poor prognosis (for review see Birchmeier and Behrens, 1994; Nollet et al., 1999). Evidence both in vivo (Perl et al., 1998) and in vitro (Frixen et al., 1991; Vleminckx et al., 1991), link E-cadherin loss with the transition to metastasis during tumor progression (for review see Yap, 1998). Interestingly, a number of recent studies indicate p120 expression is also frequently lost in tumors of the breast, prostate, colon, stomach, and bladder (for review see Thoreson and Reynolds, 2002). In many cases, p120 loss is associated with poor prognosis, suggesting that alterations in p120 expression may be important in cancer.

Despite observations of p120 loss in tumors, mutation or loss of p120 in carcinoma cell lines has not been reported. Here, we show that the p120 gene is mutated in SW48 cells and that the cadherin adhesion system is impaired as a direct

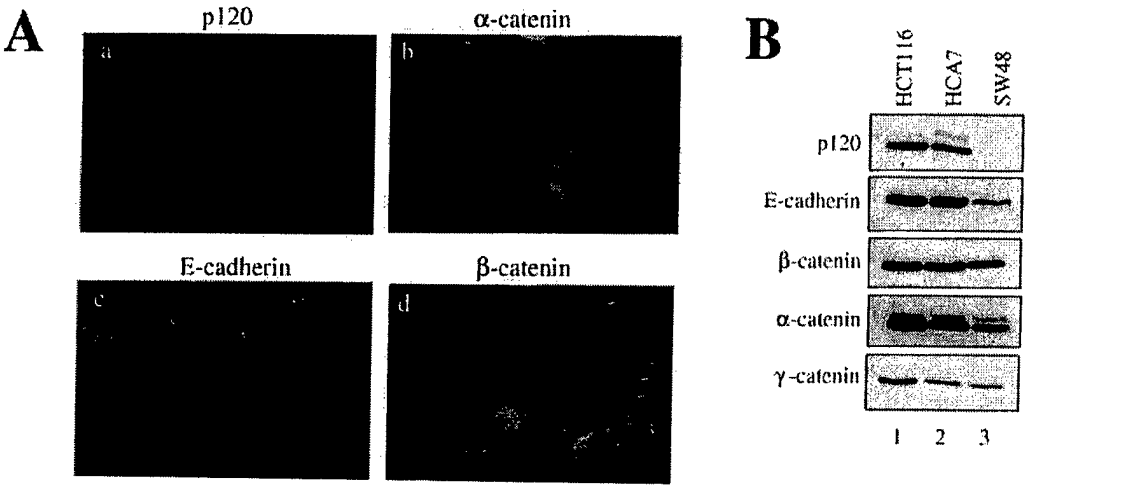

Figure 1. **Characterization of the E-cadherin complex in SW48 carcinoma cells.** (A) Members of the cadherin complex were localized in SW48 cells by immunofluorescence. SW48 cells organize aberrantly into linear arrays and do not form compact epithelial colonies. p120 was localized with mAb pp120, which binds a COOH-terminal epitope present in all known p120 isoforms. (B) Cadherin complex proteins were analyzed by Western blotting of NP-40 cell lysates derived from the colon carcinoma cell lines HCT116, HCA7, and SW48. p120 is not detected in SW48 cells by mAb pp120, indicating that the COOH terminus is absent.

consequence of p120 insufficiency. p120 is apparently essential in normal epithelial cells to directly or indirectly stabilize E-cadherin, thereby explaining its positive role in regulating E-cadherin adhesiveness.

Results

Characterization of the cadherin complex in colorectal SW48 cells

SW48 colon carcinoma cells have lost the ability to establish normal epithelial morphology. The cells form junctions, but line up end to end in loosely organized arrays that are unable to pull together into compact epithelial colonies (Fig. 1). To determine the cause of the defect, we examined the major components of the E-cadherin complex by immunofluorescence (Fig. 1 A) and by Western blotting (Fig. 1 B). Immunofluorescent analysis revealed E-cadherin staining that colocalized with α- and β-catenins at cell–cell contacts (Fig. 1 A, b–d). We initially attempted to localize p120 using well characterized monoclonal antibodies directed against the COOH-terminal epitopes (e.g., mAb's pp120, 15D2, 12F4) found on all known p120 isoforms (Wu et al., 1998). Interestingly, p120 staining was completely absent (Fig. 1 B, a). To date, this is the first observation of an adhesive cell line that does not stain with these antibodies. Consistent with these observations, analysis of SW48 cells by Western blotting showed normal levels of α-, β-, and γ-catenins (Fig. 1 B). E-cadherin levels were reduced, but significantly, p120 was not detected by mAb pp120. Although β-catenin levels are normally reduced in cells where E-cadherin expression is low, the β-catenin in SW48 cells contains a stabilizing mutation at codon 33 (Ilyas et al., 1997). This alteration probably accounts for the unexpectedly high β-catenin levels and the relatively high levels of cytoplasmic staining for both α- and β-catenins.

Mutations and splicing defects of p120 alleles in SW48 cells

The failure to detect p120 with mAb pp120 suggested possible mutations in the p120 gene. However, no alterations in the genomic organization of p120 were detected by Southern blotting. To determine if mutations were present, p120 genomic DNA from SW48 cells was sequenced by a previously established method (Keirsebilck et al., 1998). In addition, p120 cDNA clones were generated by RT-PCR from SW48 RNA. Both genomic and cloned cDNA sequences revealed a heterozygous nonsense mutation in exon 7 at nucleotide 1908 (Fig. 2 C). This C to T mutation yields a premature stop codon that truncates the protein in the third ARM repeat.

An exact molecular defect associated with the other allele could not be identified. However, RT-PCR analysis of SW48 mRNA using p120-specific oligonucleotide primers (Fig. 2 B) revealed several cDNA abnormalities, including deletion of exon 17, retention of intron 19, or retention of both introns 19 and 20 (Fig. 2 D). Long-range RT-PCR indicated that these COOH-terminal changes existed in both the 1908 C/T and the 1908 WT alleles. All of these alterations result in premature stop codons that eliminate the normal p120 COOH terminus.

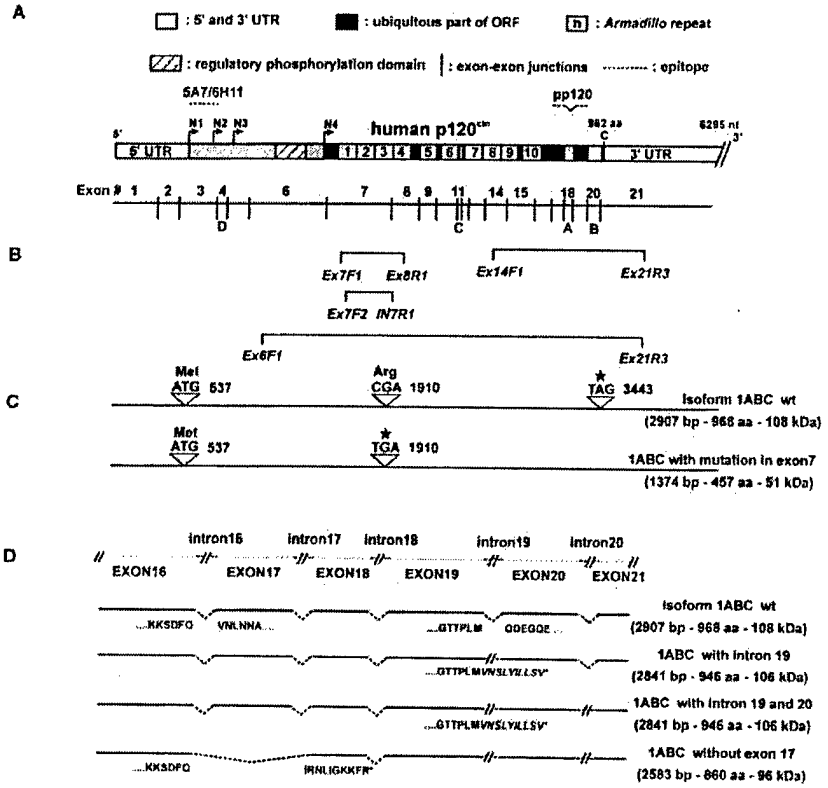

Figure 2. **Characterization of mutated p120 alleles in SW48 cells.** (A) Schematic diagram of human p120 protein and cDNA structure. N1–N4 are the four alternatively used start codons. A, B, C, and D, are alternatively spliced exons. Individual ARM repeats are numbered. Note the phosphorylation domain between N3 and N4, immediately NH_2-terminal to the ARM domain. Approximate epitopes of mAbs are indicated. (B) Primer sets used for RT-PCR and genomic DNA analysis. (C) A nonsense mutation CGA to TGA in exon 7 is indicated by the C to T mutation detected in genomic DNA and cDNA clones. The normal ATG and TAG codons are indicated. (D) Aberrant mRNA forms detected by RT-PCR. The exon structure of p120 cDNA COOH-terminal end is shown across the top. Aberrant mRNAs, each resulting in premature stop codons, are located below. Aberrant splicing in the 3′-terminal part of p120 mRNA results in the indicated gene products, which include retention of intron 19, retention of both introns 19 and 20, or exclusion of exon 17.

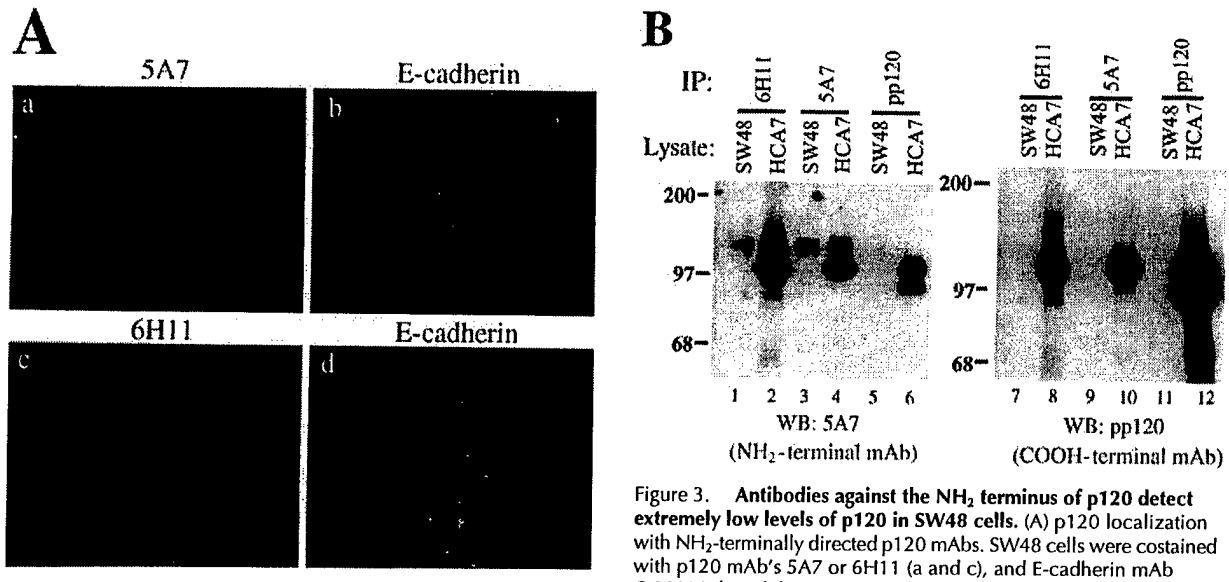

Figure 3. **Antibodies against the NH_2 terminus of p120 detect extremely low levels of p120 in SW48 cells.** (A) p120 localization with NH_2-terminally directed p120 mAbs. SW48 cells were costained with p120 mAb's 5A7 or 6H11 (a and c), and E-cadherin mAb C-20820 (b and d). (B) Quantitative analysis of p120 expression was performed by immunoprecipitating p120 from RIPA cell lysates with either NH_2-terminally directed mAbs 5A7 or 6H11, or the COOH-terminally directed mAb pp120 (top). The immunoprecipitates were split and Western blotted with mAb 5A7 (lanes 1–6) or mAb pp120 (lanes 7–12). HCA7 cells expressing normal levels of p120 were used for comparison. The blots are overexposed to visualize the extreme low levels of p120.

We then reevaluated p120 expression in the SW48 cells using monoclonal antibodies 5A7 and 6H11, which recognize different NH_2-terminal epitopes (Fig. 3). Compared with other cell lines, these antibodies revealed weak and extremely punctate junctional staining (Fig. 3 A), indicating that p120 was not completely absent in SW48 cells. Immunoprecipitation and Western blotting with mAbs 5A7 and 6H11 revealed a near full-length p120 protein, and no other bands (Fig. 3 B, lanes 1 and 3). However, the very long exposure times required to detect the mutated p120 in these experiments indicate that it is grossly underexpressed. p120 remained undetectable by mAb pp120 (Fig. 3 B, lanes 7 and 9), confirming that the normal COOH terminus of p120 is absent.

Thus, in SW48 cells, one allele of the p120 gene contains a stop codon in exon 7 (ARM repeat 3), which predicts a short product that we have not been able to detect. In addition, long-range RT-PCR experiments indicate that both alleles appear to be sensitive to abnormal alternative splicing events that eliminate the normal COOH terminus. The data explain the initial failure to detect p120 in the parental SW48 cells by several p120 mAbs directed against the COOH-terminal end. We do not yet have an explanation for the very low expression level of p120 generated from the second allele.

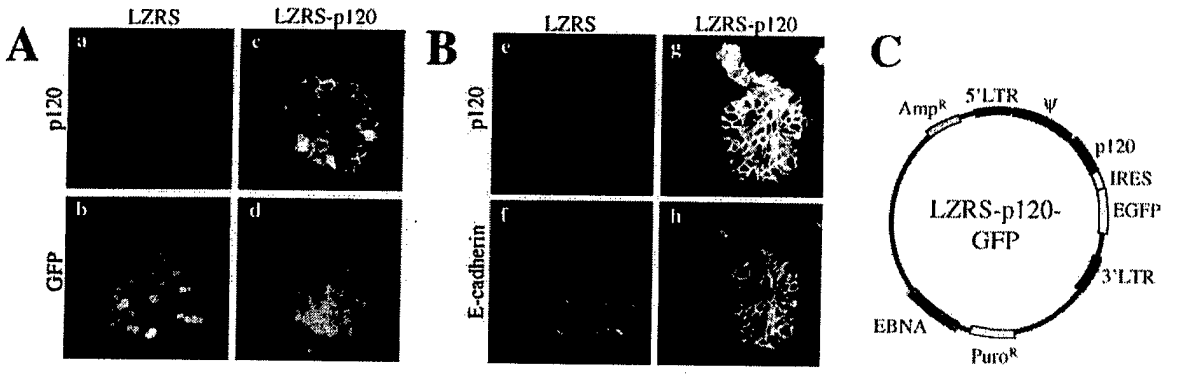

Figure 4. **p120 expression in SW48 cells rescues epithelial colony morphology.** (A and B) SW48 cells were infected with either LZRS-MS-IRES-GFP (LZRS; a, b, e, and f), or LZRS-p120-IRES-GFP (LZRS-p120; c, d, g, and h). Infected cells were sparsely plated and cultured until individual colonies appeared. (A) Rescue of epithelial morphology by p120. To visualize GFP, cells were fixed with paraformaldehyde to preserve the GFP signal (a–d). p120 was visualized with mAb 15D2 (a and c) and GFP with direct UV microscopy (b and d). GFP expression alone (b) had no effect on SW48 cell morphology. (B) Colocalization of p120 and E-cadherin in infected SW48 cells. To use the green wavelength for E-cadherin staining, cells were fixed in methanol to eliminate the GFP signal and then costained for p120 (e and g) and E-cadherin (f and h). (C) Organization of the LZRS-p120-GFP retroviral vector. An IRES is sandwiched between p120 and GFP cDNAs to allow for translation of both genes from a single mRNA transcript. Thus, the levels of GFP and p120 in infected cells are linked. Key functional elements of the vector are indicated.

Restoring WT p120 induces normal epithelial morphology

To determine whether the p120 loss or dysfunction was directly responsible for the aberrant SW48 morphology, WT-p120 expression was restored by infection with the retroviral vector pLZRS-p120-internal ribosomal entry site (IRES)-GFP. The structure of the vector is illustrated in Fig. 4 C. An IRES allows both p120 and GFP to be translated separately off the same mRNA transcript. p120 and GFP expression levels from this construct are directly proportional; cells containing high levels of GFP contain high levels of p120, and vice versa (Fig. 5 A).

Interestingly, forced expression of WT-p120 (p120ctn3A) induced a striking rescue of normal epithelial morphology. The loose organization of the parental cells (Fig. 4, A and B, b and f) gave way to compacted epithelial colonies with strong staining of p120 at cell–cell junctions (Fig. 4, A and B, c and g). Infection with pLZRS-IRES-GFP, which expresses GFP but not p120, had no effect on morphology (Fig. 4 A, a and b). These data indicate that p120 expression by itself is sufficient to restore normal epithelial morphology in these cells. Unexpectedly, p120 expression was consistently associated with significantly brighter E-cadherin staining (Fig. 4 B, compare panels f and h).

p120 epithelial rescue is dose dependent and isoform specific

Observation of individual SW48 clones after p120 retroviral infection revealed significant heterogeneity in the degree of epithelial rescue. Cells containing high levels of GFP fluorescence were consistently epithelial, whereas low levels were generally associated with the parental SW48 phenotype. Moreover, initial characterization of various p120 isoforms suggested that isoforms might vary in their ability to rescue E-cadherin function. To quantify these effects, we used gated GFP FACS to isolate polyclonal cell lines expressing low or high levels of p120 isoforms 1A, 3A, and 4A (Fig. 5). Fig. 5 A shows that the low and high GFP sorted cells express low and high levels of p120, respectively. Moreover, the different p120 isoforms in each group were expressed at similar levels, enabling accurate comparisons between isoforms.

The effects of low and high p120 expression levels on the efficiency of epithelial rescue are shown in Fig. 5 B. For each of the isoforms tested, epithelial rescue was significantly more efficient in colonies expressing high levels of p120. Interestingly, epithelial rescue by isoform 4A was much more efficient than isoforms 1A and 3A. Isoform 4A lacks the NH_2-terminal end, a region that contains the phosphorylation domain. Therefore, the sequences NH_2-terminal to the ARM domain are not required for epithelial rescue, but confer a significant negative regulatory effect on the efficiency of this process.

SW48 adhesion defect results primarily from insufficient levels of p120

Because SW48 p120 is both mutated and underexpressed, it was initially unclear which alteration was the primary cause of the phenotypic aberrations. Therefore, we asked whether the putative endogenous mutant proteins in SW48 cells could rescue epithelial morphology if we forced their expression at significantly higher levels (Fig. 6). Mutant p120 cDNAs were expressed from the LZRS-p120-IRES-GFP virus, and high expressing cell populations were isolated by FACS as described previously. The constructs tested are illustrated in Fig. 6 A. The p120 cDNA containing the C/T mutation at nucleotide 1908 was directly cloned from SW48 cells. As expected from the fact that it lacks most of the ARM domain, the product of this cDNA was inactive (Fig. 6 B, e). We also overexpressed a murine p120 mutant lacking the COOH-terminal end (ΔC), a construct very similar to the three SW48 cDNAs containing COOH-terminal splicing defects. This mutant effectively rescued compaction (Fig. 6 B, g), indicating that the COOH terminus of p120 is dispensable, provided that the cDNA is adequately expressed.

Previous work shows that high level p120 overexpression induces extensive branching of cellular processes, an effect apparently induced by inhibition of RhoA, and/or activation of Rac and Cdc42 (Anastasiadis and Reynolds, 2001). The p120 deletion mutant Δ622–628 eliminates this effect, possibly by uncoupling p120 from Rho GTPases (Anastasiadis et al., 2000). Surprisingly, expression of this mutant in SW48 cells

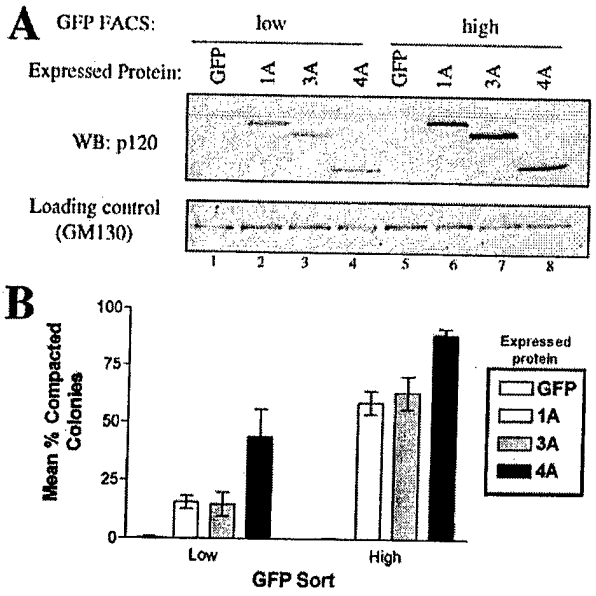

Figure 5. **Epithelial rescue by p120 is both expression level and isoform dependent.** (A) Isolation by GFP FACS of cell populations expressing low or high levels of p120 isoforms. Cells were infected with viruses containing GFP alone (lanes 1 and 5) or GFP linked by an IRES to p120 isoforms 1A (lanes 2 and 6), 3A (lanes 3 and 7), or 4A (lanes 4 and 8). Cells were sorted by FACS for low (lanes 1–4) and high (lanes 5–8) GFP expression. Protein normalization was confirmed by Western blotting with GM130 antibody. Note the tight correlation between GFP and p120 levels. Each isoform expression level is roughly equivalent within low and high level groups. (B) Quantification of the potency of p120 rescue as a function of expression level and isoform. Cells sorted as described above were plated at low density, grown into clonal colonies, and the colonies scored as compact or loose. The data reflect the ratio of compacted (rescued) colonies divided by the total number of colonies. Compacted colonies occurred more frequently when p120 is expressed at high levels. p120ctn4, which lacks the NH_2-terminal phosphorylation domain, is more efficient at inducing compaction than p120ctn1 and 3, which retain this domain.

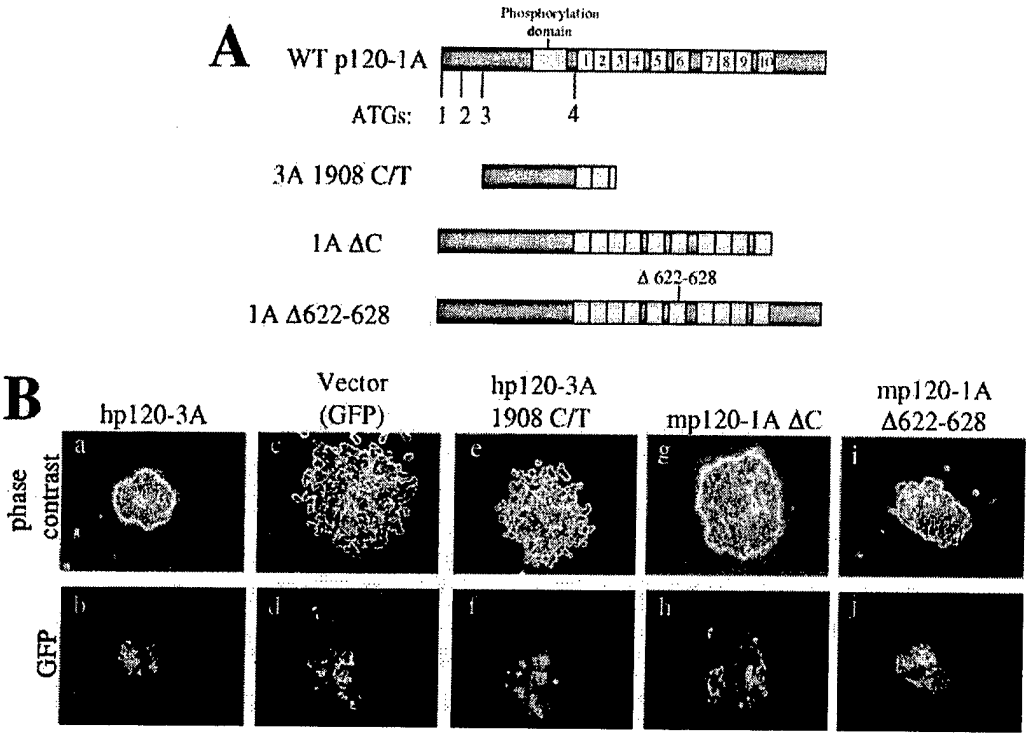

Figure 6. **The p120 COOH terminus is dispensable for epithelial rescue.** (A) Schematic of WT-p120 and p120 variants representing the mutants found in SW48 cells. Alternative ATG start sites are indicated. Light gray boxes represent ARM repeats. mp120–1A Δ622–628 contains a six-aa deletion in ARM 6 postulated to uncouple p120 from RhoA. (B) Effects of overexpression of p120 mutants in SW48 cells. Mutant constructs were cloned into pLZRS-p120-IRES-GFP and expressed in SW48 cells by retroviral infection. Infected cells were collected by gated GFP FACS, plated at low density, and colonies were photographed 1 wk later. Top panels are phase contrast images of colonies and bottom panels show GFP fluorescent images of the same cells. hp120–3A 1908 C/T does not affect the morphology (e and f), but both mp120–1A ΔC and mp120–1A Δ622–628 efficiently induce compaction (g–j).

resulted in an efficient rescue of epithelial morphology (Fig. 6 B, i), suggesting that the p120 activity required for a branching morphology is not required for epithelial compaction.

Finally, the effect of human p120ctn3A was essentially identical to that of murine p120ctn3A (Fig. 6 B, a), thus eliminating the remote possibility that the murine p120-induced phenotype was the result of species specific effects.

Together, these data indicate that the aberrant SW48 phenotype results from insufficient p120 expression rather than loss of functional information in the p120 COOH terminus. Thus, the p120-deficiency makes SW48 cells an excellent model system for p120 structure–function analysis.

Epithelial colony rescue requires p120 binding to E-cadherin

Apparently the sequences flanking the ARM repeat domain of p120 are dispensable for SW48 rescue, suggesting that the ARM domain likely contains the key functional information. To identify the critical sequences, we individually deleted the ten ARM repeats in the mouse p120ctn3A isoform and tested the constructs in SW48 cells. Infected cells were GFP sorted as described above to collect high expressing cell lines. Each of these lines expressed similar levels of p120 (Fig. 7 A, bottom). The mutant proteins were then assayed for both the ability to bind E-cadherin (Fig. 7 A) and the ability to restore epithelial morphology (Fig. 7 B). To assay the E-cadherin-p120 interaction, E-cadherin immunoprecipitates were split and Western blotted for p120 (Fig. 7 A, top) or E-cadherin (Fig. 7 A, middle). Deletion of ARM domains 1–5 and 7 disrupted the p120-E-cadherin interaction. Deletion of ARM domains 8, 9, and 10 showed a slight disruption, whereas deletion of ARM 6 had little effect.

The ability of p120 to rescue epithelial morphology correlated precisely with its ability to bind E-cadherin (Fig. 7 B). Immunofluorescent staining revealed that p120-mediated rescue was abrogated by loss of ARM repeats 1–5 and 7, but repeat 6 and repeats 8–10 were dispensable. Thus, p120 ARM repeats required for E-cadherin interaction (Fig. 7 A) are also required for rescue of epithelial morphology (Fig. 7 B).

Expression of WT p120 increases E-cadherin levels in SW48 cells by posttranscriptionally stabilizing E-cadherin

The data suggest a rescue mechanism whereby p120 stabilizes or increases E-cadherin levels in SW48 cells. To further characterize this effect, we replaced the GFP gene in LZRS-p120-IRES-GFP with the neomycin resistance gene (neo; LZRS-p120-IRES-neo) and generated clonal cell lines by G418 selection (Fig. 8 A). Lines expressing p120 levels equivalent to the control cell line HCT116 (Fig. 8 A, lane 6) were strongly compacted (unpublished data) and expressed levels of E-cadherin that were at least five fold higher than control cells (Fig. 8 A, compare lanes 4 and 5 to lanes 1–3).

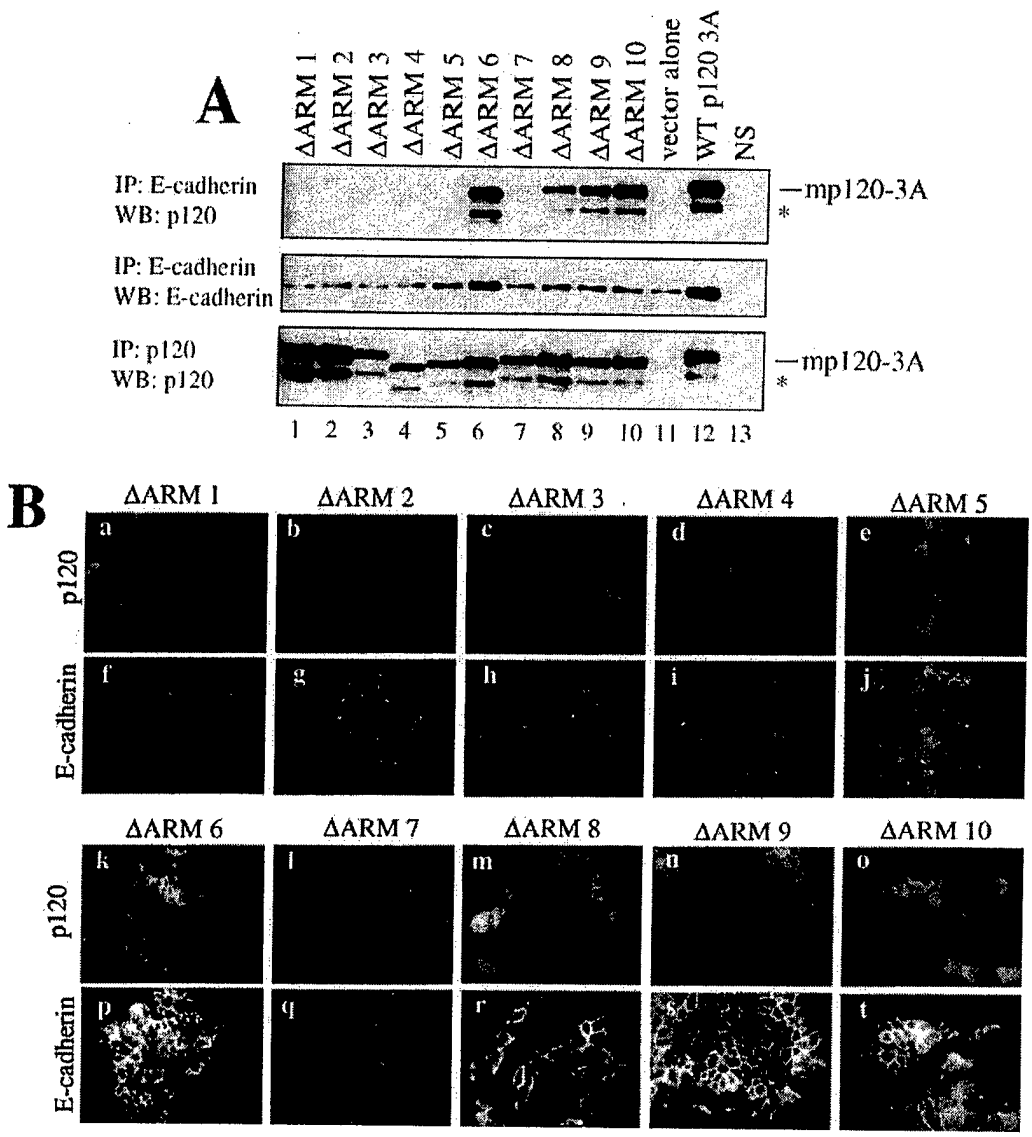

Figure 7. **p120-induced compaction is dependent on direct interaction between p120 and E-cadherin.** (A) Structure-function analysis of p120 sequences necessary for interaction with E-cadherin. Each of the ten p120 ARM repeats was individually deleted, expressed in SW48 cells, and polyclonal cell lines derived by GFP FACS. E-cadherin immunoprecipitates were isolated, divided in half, and Western blotted for E-cadherin (middle) or p120 (top). p120 immunoprecipitation and Western blot with mAb p120 controlled for the exogenous expression of p120 mutants (bottom). (*) denotes p120 degradation products. Controls include cells expressing WT-p120 (lane 12), cells infected with empty vector (lane 11), and cells immunoprecipitated with an irrelevant nonspecific antibody (NS). (B) Effects of above constructs in SW48 cells assayed by immunofluorescence for p120 (top) and E-cadherin (bottom). Deletion of ARM repeats 1–5 and 7 block rescue of compaction. ARM repeats 6 and 8–10 are dispensable. E-cadherin binding (A) correlates perfectly with the ability to rescue epithelial morphology (B).

Thus, p120 expression significantly increases the expression or stability of E-cadherin in SW48 cells.

To determine whether increased levels of E-cadherin resulted from effects of p120 on E-cadherin transcription, we assayed E-cadherin mRNA levels by Northern blotting samples obtained from parental SW48 cells and the same cells after rescue by p120 expression. Fig. 8 B shows that E-cadherin mRNA levels are unaltered by ectopic p120 expression, suggesting that p120 increases E-cadherin levels by a posttranscriptional mechanism. Interestingly, E-cadherin mRNA levels were relatively high in SW48 cells, suggesting a negative feedback loop to compensate for the low levels of E-cadherin protein. However, the increased E-cadherin levels associated with p120 overexpression did not reduce the levels of E-cadherin mRNA (Fig 8 B, lanes 2 and 3).

The effect of p120 on E-cadherin protein stability was assessed by pulse chase analysis. In clonal cell lines, ectopic expression of p120 increased the E-cadherin half-life dramatically from roughly 6 to more than 12 h (Fig. 8 C). In addition, a polyclonal population of SW48 cells, generated by LZRS-p120-IRES-neo infection and G418 selection, behaved similarly, although the stabilization was somewhat re-

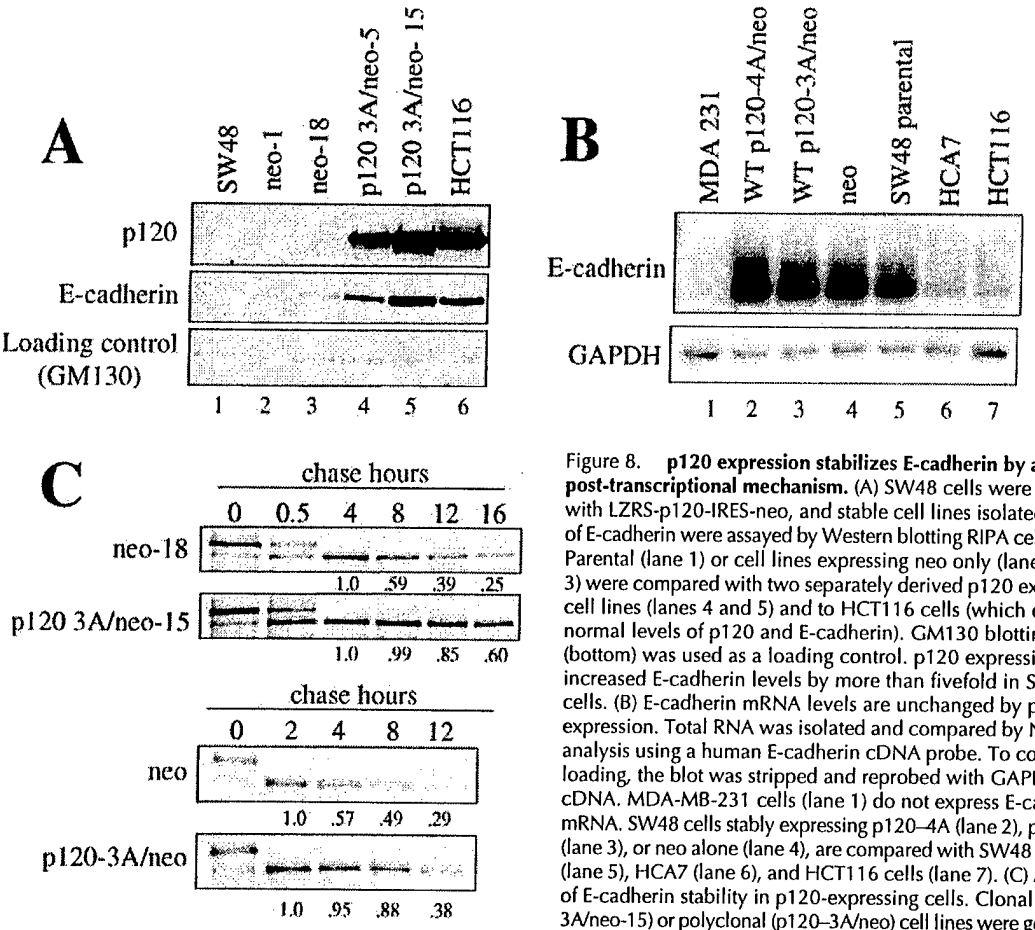

Figure 8. **p120 expression stabilizes E-cadherin by a post-transcriptional mechanism.** (A) SW48 cells were infected with LZRS-p120-IRES-neo, and stable cell lines isolated. Levels of E-cadherin were assayed by Western blotting RIPA cell lysates. Parental (lane 1) or cell lines expressing neo only (lanes 2 and 3) were compared with two separately derived p120 expressing cell lines (lanes 4 and 5) and to HCT116 cells (which express normal levels of p120 and E-cadherin). GM130 blotting (bottom) was used as a loading control. p120 expression increased E-cadherin levels by more than fivefold in SW48 cells. (B) E-cadherin mRNA levels are unchanged by p120 expression. Total RNA was isolated and compared by Northern analysis using a human E-cadherin cDNA probe. To control for loading, the blot was stripped and reprobed with GAPDH cDNA. MDA-MB-231 cells (lane 1) do not express E-cadherin mRNA. SW48 cells stably expressing p120–4A (lane 2), p120–3A (lane 3), or neo alone (lane 4), are compared with SW48 parental (lane 5), HCA7 (lane 6), and HCT116 cells (lane 7). (C) Analysis of E-cadherin stability in p120-expressing cells. Clonal (p120 3A/neo-15) or polyclonal (p120–3A/neo) cell lines were generated by infection. E-cadherin half life was ascertained by pulse-chase analysis and compared with control clonal (neo-18) and polyclonal (neo) cell lines. Chase times are indicated across the top. Densitometric analyses are located below each lane and represent a normalized value where the 4 h chase lane (top) or the 2 h chase lane (bottom) represent the value 1.0. p120 expression essentially doubles the half life of E-cadherin.

duced relative to the clonal cell line, probably because the average p120 expression level in the population is lower. Thus, p120 expression in SW48 cells substantially increases the endogenous E-cadherin half life.

Forced E-cadherin expression induces epithelial colony morphology

If p120 is acting essentially to stabilize or elevate E-cadherin levels, then forcing increased E-cadherin expression might also rescue epithelial morphology, even in the absence of p120. To test this hypothesis, we expressed WT-E-cadherin or p120-uncoupled E-cadherin (E-cad/764AAA) by infection of SW48 cells with LZRS-E-cad-IRES-neo or LZRS-E-cad/764AAA-IRES-neo followed by selection in G418 (Fig. 9). Both WT and p120 uncoupled E-cadherin rescued epithelial morphology. The rescue by E-cad-764AAA is particularly significant because the inability of this mutant to recruit p120 implies that p120 is not essential if E-cadherin levels are artificially maintained at high levels. Taken together, the data suggest that a crucial role of p120 is to regulate, stabilize, or maintain E-cadherin at levels that are high enough to generate strong adhesion.

Discussion

We describe a novel role for p120 in regulating epithelial morphology. SW48 cells are the first example of p120 mutation in cancer. More importantly, p120 is grossly underexpressed in these cells, providing the first opportunity to examine the effects of p120 loss and reconstitution in vitro. The striking rescue of SW48 epithelial morphology by expression of WT-p120 argues strongly that p120 deficiency is the critical factor limiting adhesiveness and epithelial morphology in these cells. The ability of the endogenous E-cadherin to function efficiently in the presence of ectopic p120 indicates that the endogenous E-cadherin itself is fully functional, albeit underrepresented in the absence of p120. Thus, the low levels of E-cadherin in SW48 cells are likely secondary to the root problem of p120 insufficiency. A key observation is that E-cadherin levels increased substantially upon reconstitution of p120 due to a doubling of the E-cadherin half life. Moreover, the specific p120 ARM repeats required for interaction with E-cadherin were also necessary for reactivation of the cadherin system. Thus, p120 stabilizes E-cadherin and rescues its function via a mechanism dependent on their direct interaction.

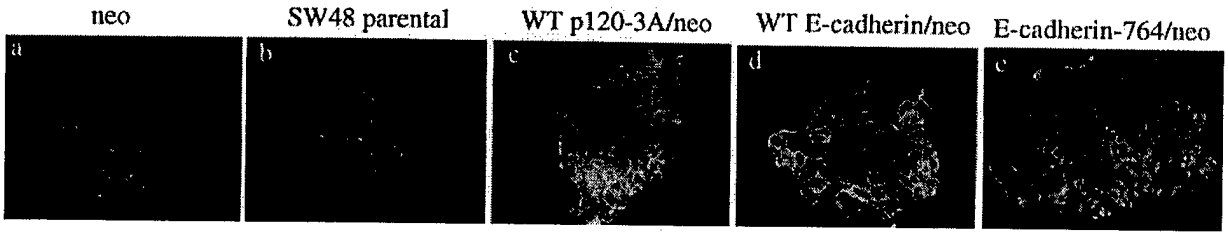

Figure 9. **Epithelial rescue by overexpression of WT or p120-uncoupled E-cadherin in SW48 cells.** SW48 cells were infected with LZRS-neo viruses containing p120, E-cadherin, or p120-uncoupled E-cadherin (E-cadherin-764), and colonies generated by G418 selection. Cells are stained for immunofluorescence using E-cadherin mAb C-20820. Expression of neo alone (a) had no effect on the parental SW48 phenotype (b). However, WT-E-cadherin (panel d) and p120-uncoupled E-cadherin (e) induced an epithelial morphology similar to that induced by p120 expression (c).

The observations reported here are based on SW48 cells but are probably generally applicable. For example, significantly increased E-cadherin staining is observed in MDCK cells overexpressing either p120 (unpublished data), or δ-catenin (Lu et al., 1999), a close structural and functional relative of p120. However, in normal cells such as MDCK, the significance of this effect is not obvious because the cells already possess epithelial morphology and high levels of p120 and E-cadherin. In contrast, the effects of p120 overexpression in SW48 cells are striking due to the initial condition of p120 deficiency, the resulting low levels of endogenous E-cadherin, and the diffuse cellular morphology.

Evidence in the literature supports several possible explanations for these observations. The simplest is that the p120 interaction with E-cadherin is sufficient to physically stabilize the E-cadherin complex. Crystallographic studies indicate that the cadherin cytoplasmic domain is essentially unordered in the absence of binding partners (Huber et al., 2001). Alternatively, p120 may competitively block interactions of E-cadherin with other factors that promote E-cadherin recycling or degradation. Endocytosis has been proposed as a mechanism for down-regulating or recycling cadherins (Le et al., 1999) and is postulated to play a crucial role in the dynamic regulation of adhesiveness. The E3 ubiquitin-ligase Hakai interacts with the E-cadherin JMD in a tyrosine phosphorylation-dependent manner, thereby promoting ubiquitination and endocytosis of the complex (Fujita et al., 2002). Because multiple tyrosine kinases and phosphatases have been directly or indirectly linked with the cadherin complex (for review, see Daniel and Reynolds, 1997), the loss of p120 in SW48 cells may leave the E-cadherin JMD exposed to increased tyrosine phosphorylation resulting in Hakai-mediated endocytosis. PS1 also competes with p120 for binding the cadherin JMD (Baki et al., 2001), and although one report suggests that PS1 stabilizes the cadherin complex (Baki et al., 2001), a follow-up study suggests that PS1 may promote E-cadherin degradation by specific proteolytic cleavage events (Marambaud et al., 2002). Thus, p120 binding may block such interactions, and in the absence of p120, these proteins might act constitutively in SW48 cells to reduce the amount of E-cadherin.

Another possibility is that p120 may possess an activity that promotes stability of the cadherin complex. For example, recent evidence indicates that p120 can inhibit RhoA and activate Rac and Cdc42, known modulators of the actin cytoskeleton (for review see Anastasiadis and Reynolds, 2001). Although these reports focus on the regulation of GTPases by a soluble pool of p120, they also suggest the possibility that p120 recruits and coordinates the actions of these proteins in the cadherin complex. These GTPases may be necessary for cadherin clustering (Braga et al., 1997; Takaishi et al., 1997; Jou and Nelson, 1998). Thus, p120 binding to E-cadherin may promote the local assembly and/or organization of the underlying actin cytoskeleton resulting in stabilization of the adherens junction. On the other hand, our preliminary data (Fig. 6 B, i) show that a mutant (Δ622–628), presumably RhoA uncoupled and unable to induce cellular branching (Anastasiadis et al., 2000), effectively rescues epithelial morphology in SW48 cells. Thus, it is unclear whether p120's branching activity, and/or the p120/Rho GTPase connection is relevant to the observed epithelial rescue in SW48 cells.

Because p120-uncoupled cadherins can restore epithelial morphology in several systems (Aono et al., 1999; Thoreson et al., 2000; this work, Fig. 9), it has been suggested that cadherins can function efficiently in the absence of p120 (Aono et al., 1999). Our data provide a cautionary note on the interpretation of experiments using p120-uncoupled cadherins based on the fact that these experiments rely on ectopic cadherin expression driven by constitutively active promoters. If stabilizing E-cadherin is an important role for p120 under normal circumstances, then constitutive overexpression of p120-uncoupled cadherins may at least partially compensate for p120 function despite the absence of p120 binding. Indeed, overexpression of p120-uncoupled E-cadherin can rescue epithelial morphology in SW48 cells, but the absence of p120 in the parental cell line clearly leads to malfunction of the cadherin system. Moreover, the known p120-uncoupling mutations simultaneously eliminate binding sites for p120, Hakai, and PS1, thereby potentially abolishing both cadherin stabilizing and destabilizing mechanisms. These possibilities highlight the significance of our observations in SW48 cells, which directly address the consequences of p120 loss and reconstitution.

Based on experiments in Colo205 cells, it was suggested that p120 negatively regulates cadherin adhesiveness (Aono et al., 1999). These cells normally express all members of the cadherin complex, including p120, but are nonetheless poorly adhesive (Aono et al., 1999). An NH_2-terminally truncated p120 mutant (similar to our p120ctn4 isoform) strongly reactivates the cadherin system in these cells,

whereas WT-p120 has no effect (Aono et al., 1999). Thus, the cadherin system in Colo205 cells appears to be constitutively inactivated through a signaling mechanism requiring the phosphorylation domain of p120. In contrast, we find that both WT and NH_2-terminally truncated p120 (p120ctn4) reactivate cadherin function in SW48 cells. These contrasting scenarios suggest that the principle defect in SW48 cells is p120 insufficiency, whereas the principle defect in Colo205 cells involves aberrant signaling through p120. Although these cell lines represent extreme cases, it is possible that both scenarios exist to various degrees in many tumors and may contribute to cadherin malfunction in malignancy. It is worth noting that the p120 NH_2-terminal domain had a negative effect on adhesion in SW48 cells (as also reported in Colo205), but the effect was considerably less prominent than the inhibition observed in Colo205 cells. In light of our data, we suggest that p120 is principally a positive regulator of adhesion. The regulatory region containing the phosphorylation domain is likely to act like a dimmer switch and provides a mechanism for signaling pathways to modulate cadherin functions, such as adhesion and motility, through posttranslational modification of p120.

The data support a basic model whereby the positive role of p120 in adhesion requires its interaction with E-cadherin and results in subsequent stabilization of the complex. The regulatory region then acts as a dimmer switch, potentially controlled by various signaling events resulting in phosphorylation and/or dephosphorylation of specific p120 residues. These actions may control the on/off rate of p120, whose affinity for cadherins appears to be relatively weak (Reynolds et al., 1994; Shibamoto et al., 1995; Staddon et al., 1995). Conditions that promote p120 binding would therefore be expected to increase adhesiveness. Cytoplasmic p120 may promote cell motility through action on Rho GTPases (Noren et al., 2000; Grosheva et al., 2001). Thus, p120 translocation to the cytoplasm could be important for other events typically associated with E-cadherin downregulation, as suggested previously (Anastasiadis and Reynolds, 2001). Alternatively, p120 phosphorylation may control a specific p120 activity that functions within the cadherin complex (e.g., recruitment of Rho GTPases or as yet unidentified binding partners), which in turn may be important for dynamic modulation of adhesion as occurs in motile cells. The various mechanisms proposed are not mutually exclusive and the end result is likely influenced by several events that act together.

Accumulating evidence indicates that p120 expression is frequently altered in human carcinomas. Quite striking are reports in which p120 staining is completely absent from certain tumors of the breast, stomach, prostate, bladder, and colon (for review see Thoreson and Reynolds, 2002). In light of these data, it is unclear why p120-deficient epithelial cell lines are not common. Nonetheless, these observations suggest that p120 function may be important in cancer and raise new questions as to how p120 expression is regulated.

E-cadherin downregulation is common in tumors and occurs through several mechanisms, including mutation (for review see Berx et al., 1998; Berx and van Roy, 2001), promoter methylation (Matsumura et al., 2001), and regulatory alterations mediated by various transcription factors (Batlle et al., 2000; Cano et al., 2000; Comijn et al., 2001). We suggest a new mechanism based on the ability of p120 to posttranscriptionally stabilize E-cadherin. In some tumors, p120 loss may be an initial event leading to reduced levels of E-cadherin. Since E-cadherin stabilizes α- and β-catenin (De Leeuw et al., 1997), one consequence of p120 downregulation may be reduced levels of all members of the cadherin complex, and thus an overall reduction in cadherin activity. Ironically, β-catenin is not downregulated in SW48 cells because of a stabilizing mutation at codon 33 (Ilyas et al., 1997).

In conclusion, we describe the first example of a p120-deficient carcinoma cell line and a novel role for p120. p120 insufficiency in SW48 cells appears to be directly responsible for a significant reduction in the amount of E-cadherin and for the associated defects in epithelial morphology. The residual p120 levels in these cells may very well be essential for the fact that even limited junctions were observed. Restoring p120 substantially increased E-cadherin levels and rescued epithelial morphology by mechanisms that were completely dependent on the direct interaction between these proteins. We believe this activity to be responsible for the positive role of p120 in adhesion and necessary to maintain E-cadherin abundance and activity at levels sufficient to mediate strong cell–cell adhesion. Our data also suggests that the NH_2-terminal domain contains regulatory sequences necessary for dynamic modulation of this otherwise positive action. Based on these observations, we also suggest that p120 loss in tumors may induce the subsequent downregulation of E-cadherin to levels that cannot support normal epithelial morphology.

Materials and methods

Cell culture

SW48 cells were grown in DME/F-12 with 1% L-glutamine. All other cells were grown in DME. All media was supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO BRL). Phoenix 293 cell media contained heat-inactivated FCS.

Immunofluorescence

5×10^3 cells per coverslip were grown 7–10 d before immunofluorescent labeling as previously described (Mariner et al., 2000). To eliminate GFP, cells were fixed in 100% methanol for 7 min at −20°C. To visualize GFP expression, cells were fixed in 3% paraformaldehyde for 30 min and permeablized with PBS/0.2% Triton-X for 5 min. Primary antibodies were used at: p120, 0.5 μg/ml (Transduction Labs); anti–β-catenin, 1/1,000 (C-2206; Sigma-Aldrich); anti–α-catenin, 1/1,000 (C-2081; Sigma-Aldrich); anti–E-cadherin, 1/2,500 (C-20820; Transduction Labs); p120 mAbs 5A7 and 6H11, 2 μg/ml (Wu et al., 1998); and hECD1, 1 μg/ml. Secondary antibodies goat anti–mouse IgG2a Alexa 488, goat anti–mouse IgG1 Alexa 594, goat anti–mouse Alexa 488, and goat anti–rabbit Alexa 594 (Molecular Probes) were diluted 1:600.

Immunoprecipitation and Western blotting

Immunoprecipitation and Western blot procedures have been described previously (Reynolds et al., 1994). For coimmunoprecipitation, cells were lysed in CSK buffer (10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, and 0.5% NP-40). RIPA and NP-40 whole cell lysates were prepared as in (Mariner et al., 2000). Protein was quantitated by BCA Protein Assay (Pierce Chemical Co.). Antibodies pp120, hECD1, anti–β-catenin, anti–α-catenin, and peroxidase-conjugated donkey anti–mouse and mouse anti–rabbit secondaries (Jackson ImmunoResearch Laboratories) were used as described in (Thoreson et al., 2000). Other antibody concentrations were: Plakoglobin mAb I5F11, 2.5 μg/ml; p120 mAb 5A7, 2 μg/ml; and GM130, 1:250 (610823; Transduction Labs).

Viral production, infection, and production of stable cell lines

Phoenix 293 cell transfection (Grignani et al., 1998) was performed as described on the Nolan lab website (http://www.stanford.edu/group/nolan/),

incubating for 7–9 h at 37°C. Cells were selected with 5 μg/ml puromycin (Sigma-Aldrich) and passaged once before viral harvest. To harvest virus, cells at 75% confluence were incubated for 16 h at 37°C. Collected media was passed through a 0.45-μm syringe filter (Pall Corporation) and immediately used or stored at −70°C. To infect, 4 μg/ml polybrene (Sigma-Aldrich) was added to virus stock before overlaying cells plated at 6×10^4 cells/60-mm dish or 1.8×10^5 cells/100-mm dish. Cells were incubated overnight and replated in SW48 media. To produce clonal stable cell lines, cells were infected with the LZRS-MS-IRES-neo virus containing the genes of interest, sparsely plated, selected with 875 active units/mL Geneticin (GIBCO BRL) for 7–10 d, and isolated using cloning cylinders.

FACS

Cells infected with LZRS-MS-IRES-GFP virus containing the genes of interest were split 1:1, grown 24 h at 37°C, and suspended at 2×10^6 cells/ml in PBS (15 mM NaCl, 10 mM NaH_2PO_4). GFP gates were set to collect the cells in the bottom third (low expressors) or top third (high expressors) of the GFP range (488 nm) using a FACStar Plus cell sorter. Gating parameters were predetermined by observing GFP levels in pilot experiments.

Quantitative analysis of epithelial rescue

Cells expressing p120 isoforms were sorted into high and low GFP expressing populations. 5×10^3 cells/90-mm dish were grown for 7 d. The number of colonies out of 100 that exhibited the compacted colony phenotype were counted. GraphPad Prism software was used for statistical (Bonferroni's Multiple Comparison Test) and graphical analysis. Results reflect pooled data from three independent sorting experiments and show standard deviation of the means.

Northern analysis

Total RNA was isolated with TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. 20 μg of each RNA sample was analyzed by Northern blotting with an E-cadherin probe generated by PCR-amplification of a 1,326-bp fragment of human E-cadherin using 5′-TGATGTGAACACCTACAATGCCG-3′ and 5′-CCTCCGAAGAAACAGCAAGAGC-3′ as primers. Probing with full-length murine GAPDH controlled for loading. Probes were α-P^{32} dCTP-labelled by primer extention (Prime-It II Kit; Stratagene). Nylon membranes were hybridized using Express Hyb hybridization solution (CLONTECH Laboratories, Inc.) following the manufacturer's protocol. Results were visualized by autoradiography using Phosphor Screens (Molecular Dynamics) and a PhosphoImager 445-SI (Molecular Dynamics).

Cloning

pLZRS-MS-IRES-GFP and pLZRS-MS-IRES-neo retroviral vectors were generated by replacing the lacZ gene of pLZRS-lacZ with EGFP or neo genes (from pIRES2-EGFP and pIRES-neo; CLONTECH Laboratories. Inc.). A shuttle vector (pMS) was created by replacing the pBluescript (Stratagene) polylinker with a polylinker containing SgfI, EcoR1, AfeI, Kpn1, and SfiI restriction sites. The same polylinker was inserted into LZRS-IRES-MS-GFP (or neo). All constructs were made by cloning cDNA into pMS, then moving it to LZRS by a SgfI-SfiI ligation. LZRS-hp120-3A-1908-C/T-GFP was generated from cDNA cloned directly from SW48 mRNA. All other deletion mutants were created using ExSite Mutagenesis (Stratagene). The deleted sequences in the LZRS-mp120-GFP constructs are based on the aa numbering system for p120-1A (Anastasiadis and Reynolds, 2000). Listed aa are inclusive of the deletion. ΔC, (aa 831–932); ΔARM1, (aa 358–400); ΔARM2, (aa 401–443); ΔARM3, (aa 445–486); ΔARM4, (aa 487–545); ΔARM5, (aa 546–591); ΔARM6, (aa 592–649); ΔARM7, (aa 650–696); ΔARM8, (aa 697–737); ΔARM9, (aa 738–787); ΔARM10, (aa 788–824). All ARM repeat domain mutants were made in mp120 3A. mp120-1A Δ622-628 and p120 uncoupled E-cadherin (E-cad-764AAA) have been described previously (Anastasiadis et al., 2000; Thoreson et al., 2000). The p120-4A isoform was generated from p120-3A by using PCR to eliminate the first three start codons and starts at aa 324.

RT-PCR and genomic sequencing

For RT-PCR analysis, RNA was isolated from human tumor cell lines using the RNeasy kit (QIAGEN). cDNA was synthesized using Superscript™ II Rnase H⁻ reverse transcriptase (GIBCO BRL), incubated with 0.5 U Rnase H (GIBCO BRL), and PCR amplified using Taq DNA polymerase (GIBCO BRL). Products were isolated using a Concert Rapid Gel extraction kit (GIBCO BRL) and directly sequenced or cloned in the pGEM®-T cloning system (Promega). Primers EX7F1, EX8R1, EX14F1, EX21R3, and EX6F1 were used and have been previously described (Keirsebilck et al., 1998). Intron-exon boundaries were determined by partial sequence analysis of subcloned fragments containing protein-encoding sequences (Keirsebilck et al., 1998). Intron-specific amplimers were used to screen all the p120-specific exons for relevant mutations. As exon 7 contains 464 nucleotides, two primer pairs were used. The mutation was detected using primers EX7F2 (5′-GTCAAGTCCAATGCAGCTGCATA-3′) and IN7R1 (5′-ATCCCTCTAGTTCTCAACATCAC-3′) by PCR using Goldstar DNA polymerase (Eurogentec) followed by sequencing.

Pulse chase

Pulse chase analysis was performed as described (Fujita et al., 2002). Cells were lysed in RIPA buffer and E-cadherin immunoprecipitated with 2 μg of hECD-1 mAb. Prior to drying, gels were fixed and treated with Amplify (Amersham Biosciences) according to manufacturer's protocol. Radioactive bands were visualized by autoradiography with phosphorscreens as described above and quantified using ImageQuant (Molecular Dynamics).

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REVIEW

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Altered expression of the catenin p120 in human cancer: implications for tumor progression

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Abstract Tumor progression in epithelial tissues is characterized by a series of genetic and epigenetic changes that lead ultimately to metastasis. Alterations in E-cadherin and its cytoplasmic regulators, the catenins, have been implicated as central to this process. Here, we focus on p120-catenin and its rising incidence in the pathology literature as a molecule altered in human tumors. The data show that p120 is frequently altered and/or lost in tumors of the colon, bladder, stomach, breast, prostate, lung, and pancreas. Moreover, in some cases p120 loss appears to be an early event in tumor progression, possibly preceding loss of E-cadherin. Potential roles of p120 as a tumor suppressor or metastasis promoter are discussed.

Key words p120ctn · p120 · cadherin · catenin · microenvironment · tumor suppressor · metastasis

Introduction

Interactions between the tumor and its microenvironment may play an important role in the regulation of epithelial (E)-cadherin expression and function during tumorigenesis and metastasis. E-cadherin, the major cell–cell adhesion protein in epithelial tissues, is frequently downregulated in epithelial cancers (reviewed in Birchmeier and Behrens, 1994). In gastric and lobular breast cancers, the E-cadherin gene is directly mutated very early in the genesis of the tumor, indicating a tumor suppressor role (reviewed in Berx and Van Roy, 2001). In most other epithelial cancers, E-cadherin is downregulated as a late event and is believed to mark the transition to metastasis. In the latter cancers, downregulation has been linked to a variety of potentially reversible epigenetic events, which include chromatin rearrangements, promoter methylation, and alterations in transcriptional regulation (reviewed in Berx and Van Roy, 2001). Thus, E-cadherin is widely acknowledged as both a tumor and metastasis suppressor, and the search for strategies to repress metastasis have led to intense study of the mechanisms and molecules regulating E-cadherin function (reviewed in Takeichi, 1995; Yap, 1998).

E-cadherin function is also regulated by cytoplasmic binding partners called catenins (α-catenin, β-catenin, and p120-catenin/p120). Cancer-related alterations in α- and β-catenins have been reviewed elsewhere (Nollet et al., 1999). p120 was originally identified as a Src substrate, (Reynolds et al., 1992, 1989), and later as a major cytoplasmic binding partner for members of the cadherin superfamily of cell–cell adhesion molecules (Reynolds et al., 1994; Shibamoto et al., 1995; Staddon et al., 1995). p120 binds to the so-called "Juxtamembrane domain" (JMD) of E-cadherin, where along with other catenins, it is thought to regulate E-cadherin's adhesive interactions between cells (Aono et al., 1999; Thoreson et al., 2000; Yap et al., 1998). p120 has been implicated in the mechanism of cadherin clustering, possibly through regulation of Rho-GTPases (Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000; reviewed in Anastasiadis and Reynolds, 2001). Like β-catenin, its cousin in the cadherin complex, p120 can also translocate to the nucleus, although its role at this location is unknown (Daniel and Reynolds, 1999; van Hengel et al., 1999). These issues have been reviewed in detail recently (Anastasiadis and Reynolds, 2000, 2001).

Most proteins physically or functionally related to p120 and/or the E-cadherin complex are oncogenes or tumor suppressors (e.g. Src-family kinases, receptor tyrosine kinases, E-cadherin, β-catenin, Wnt-1, APC, etc.).

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Although mechanistic studies support such a role for p120, the relationship of p120 to human cancer is not yet clear. Potentially important clues have appeared recently in the pathology literature. In the first such report, p120 staining was absent from 3 out of 13 colon carcinomas examined (Skoudy et al., 1996). Follow-up studies in colon (Gold et al., 1998) and breast (Dillon et al., 1998) confirmed that p120 is indeed lost in some tumors, and that p120 loss is in many instances associated with poor prognosis (see below). Abnormalities in p120 expression have now been reported in colorectal, bladder, gastric, breast, prostate, lung, pancreatic, melanoma, and endometrial tumors (see Table 1). These observations are striking given that E-cadherin and p120 are invariably present at high levels in normal epithelial tissue. Below, we review the pathology literature on p120 and attempt to interpret the data in light of mechanistic studies suggesting roles for p120 as a tumor suppressor and/or a metastasis promoter.

Altered p120 expression in human tumors

Colon carcinoma

p120 has been examined most often in colorectal tumors, where its expression is altered in the majority of cases. In a small study of 13 human colorectal tumors, p120 was decreased in 69% of tumors (9/13), and this reduction significantly correlated with a larger tumor size (Skoudy et al., 1996). In three cases, staining was defined as negative (fewer than 10% of cells expressing). In general, abnormal expression patterns of p120 and E-cadherin were correlated, suggesting coordinate regulation of p120 and E-cadherin. In a larger study of 44 primary colorectal tumors (Gold et al., 1998), p120 levels were decreased in 86% of cases. In 18% of cases there was regional loss of p120, which correlated with high-stage disease, nodal metastasis, and decreased survival. A third report of 43 colorectal cancers revealed altered p120 staining in 65% of cases (Karayiannakis et al., 1999). p120 was absent in 21%, cytoplasmic with a loss of membranous staining in 25%, and heterogeneous in 19%, but there was no correlation between p120 expression and tumor grade or stage. Interestingly, one study found decreased p120 levels in 100% (20/20) of hyperplastic colorectal polyps, suggesting that p120 changes may occur early during tumor progression (Valizadeh et al., 1997). Overall, these studies suggest that p120 reduction and/or loss is common in colorectal tumors. Thus, p120 loss may be associated with disease progression, an observation consistent with its proposed role as a tumor suppressor.

Bladder carcinoma

In an early report of 48 bladder tumors, 15 tumors showed heterogeneous p120 staining, while three displayed negative staining (defined by a complete absence of immunoreactivity) (Shimazui et al., 1996). Heterogeneous or absent p120 expression correlated with increased tumor grade and stage, and poor survival. E-cadherin and other catenins were also examined, and discrepancies between E-cadherin and p120 expression were seen more often than with other catenins, suggesting a lack of coordinate regulation. Similarly, another study reported abnormal p120 expression (heterogeneous, cytoplasmic, or negative) in 57/68 (84%) of bladder tumors (Syrigos et al., 1998). These changes also correlated with increased grade and stage and with poor survival. For example, 30/31 grade III tumors and 16/17 stage 4 tumors showed p120 abnormalities. Finally, in a large study of 102 bladder tumors, abnormal p120 expression occurred in 70% of cases (Nakopoulou et al., 2000). No specimens displayed loss of β-catenin staining, but p120 was completely absent (staining in <10% of cells) in 17 cases. p120 and E-cadherin expression correlated in 94% of cases, while 6% displayed a reduction in p120 with no alteration in E-cadherin. In addition, simultaneous abnormal expression of E-cadherin, p120, and β-catenin correlated with high grade and decreased survival. The extremely high levels of p120 decrease and/or loss in bladder tumors are consistent with a tumor suppressor role in this tissue.

Gastric carcinoma

Three studies have investigated p120 in gastric tumors. The first examined 40 tumors and found altered expression in 70% (28/40) (Karayiannakis et al., 1999). p120 was absent in 18%, heterogeneous (having areas of mixed positive and negative cells) in 15%, and cytoplasmic with a loss of membranous staining in 37%. There was no correlation with tumor grade or stage. In a second study, p120 staining was reduced in over half of 36 gastric tumors (Karatzas et al., 2000). Changes in E-cadherin expression directly correlated with those of p120 and of α-, β-, and γ-catenins.

Conversely, a third study of gastric carcinomas reported mostly strong cytoplasmic p120 staining with reduced localization at the membrane (Jawhari et al., 1999), which correlated with E-cadherin loss. This situation is similar to results observed in cultured cells (Thoreson et al., 2000), where cadherin loss is associated with translocation of p120 from the membrane to the cytoplasm. Under these circumstances, cytoplasmic p120 appears to be stable, and thus differs from α- and β-catenins, which are efficiently degraded in the absence of a cadherin binding partner (Nagafuchi et al., 1991; Papkoff, 1997). Interestingly, the Jawhari study showed striking examples where strong cytoplasmic p120 staining and near complete p120 loss were observed in different parts of the same tumor section. This apparent "internal control" suggests that both results reflect the

Table 1 Status of p120 in human tumors

Tumor type	Summary	References
Colorectal	Altered in 65% (28/43) (*absent in 21% (9/43)**, cytoplasmic in 25% (11/43), heterogeneous in 19% (8/43))	Karayiannakis et al., 1999
Colorectal	Decreased or lost in 86% (38/44) (*loss in 18% (8/44)* correlates with stage, nodal metastases, and decreased survival; decreased in 30/44 (68%))	Gold et al., 1998
Colorectal	Decreased in 9/13 (*absent in 23% (3/13)*, cytoplasmic in 46% (6/13) (diminished and mainly in cytosol); decrease correlates with larger tumor size)	Skoudy et al., 1996
Colorectal polyps	Decreased in 100% (20/20) of hyperplastic polyps; heterogeneous in 20% (4/20) of adenomatous polyps; reduced in 20% (4/20) of inflammatory polyps	Valizadeh et al., 1997
Bladder	Decreased in 70% (71/102). *Absent in 17% (17/102)*; reduction correlates with increased grade and stage.	Nakopoulou et al., 2000
Bladder	Altered in 84% (57/68) (correlates with increased grade and stage and with poor survival)	Syrigos et al., 1998
Bladder	Decreased or absent in 38% (18/48) (*absent in 6% (3/48)*, heterogeneous in 31% (15/48); both correlate with tumor grade, stage, and poor survival)	Shimazui et al., 1996
Gastric	Decreased in 56% (20/36)	Karatzas et al., 2000
Gastric	Altered in 70% (28/40) (*absent in 18% (7/40)*; cytoplasmic in 37% (15/40), heterogeneous in 15% (6/40))	Karayiannakis et al., 1999
Gastric	Altered in 66% (45/68) (upregulation of cytoplasmic p120 in 66% (45/68), loss of membrane expression in 32% (22/68))	Jawhari et al., 1999
Breast	Abnormal in 73% (58/80), assoc. with loss of progesterone receptor (*negative in 10% (8/80)*, cytoplasmic in 5% (4/80), heterogeneous in 58% (46/80))	Nakapoulou et al., 2002
Breast	Abnormal in 50% (*absent in 10%*, altered in 40%)	Dillon et al., 1998
Prostate	Decreased in 49% (55/112), correlates with increased grade, stage, and ploidy	Kallakury et al., 2001a
Prostate	Decreased in 45% (53/118), correlates with increased grade, stage, ploidy, and serum PSA	Kallakury et al., 2001b
Lung	Decreased in 94% (*negative or low in 61%*, intermediate expression in 33%; decrease correlates with stage, tumor size, and invasion)	Bremnes et al., 2002
Pancreatic	Altered in 60% (*absent in 15% (3/20)*, cytoplasmic in 25% (5/20), heterogeneous in 20% (4/20))	Karayiannakis et al., 1999
Melanoma	Heterogeneously expressed; *frequently absent*	Zhang and Hersey, 1999
Endometrial	Abnormal localization in 100% (10/10) of poorly differentiated tumors	Miyamoto et al., 2000
Renal	No changes	Kuroiwa et al., 2001

*Italics are added to highlight the frequent reports of p120 loss in human tumors

true status of p120 in these adjacent tumor regions. The cytoplasmic p120 observed in this study is thus consistent with what might be expected from cell culture studies, but differs from results of other gastric tumor studies.

Thus, p120 in gastric tumors is often lost, or found at high levels in the cytoplasm, depending on the study. Strong correlations between p120 changes and tumor progression in stomach cancer were not reported.

Breast carcinoma

Two studies have examined p120 expression in breast cancer. In a study of 91 invasive ductal carcinomas, p120 was completely lost in 10% of cases (Dillon et al., 1998). Complete loss was defined as complete absence of antibody reactivity in a particular region of the tumor, and the result was confirmed with several different monoclonal antibodies. p120 loss contrasted with α- and β-catenin staining, which was sometimes altered but never completely absent. There was no correlation between the expression of p120 and that of E-cadherin or α- and β-catenins. A second report on 80 invasive breast carcinomas also showed p120 loss in 10% of cases (Nakopoulou et al., 2002). In addition, 58% (46/80) demonstrated heterogeneous expression, while only 5% showed cytoplasmic staining. Simultaneous abnormal expression of p120, E-cadherin, α-catenin, and β-catenin was seen in 41% of cases. Of these, only abnormal p120 expression was significantly associated with loss of progesterone receptor. Thus, both studies suggest that a portion of invasive breast carcinomas undergo complete loss of p120, but disagree as to whether p120 loss is linked to loss of other proteins in the cadherin complex.

Prostate carcinoma

In the first description of p120 in prostate, p120 was examined in 112 adenocarcinomas (Kallakury et al., 2001a). Decreased expression was noted in 49% (55/112), and this correlated with tumor grade, stage, and ploidy. Decreased p120 levels correlated with decreased levels of E-cadherin, α-catenin, and CD44. Reduced CD44 levels correlated with increased preoperative serum PSA levels, an early marker for prostate cancer. In a similar report of 118 prostate adenocarcinomas,

p120 expression was decreased in 45% of tumors. This decrease also correlated with tumor grade, stage, and ploidy (Kallakury et al., 2001b). A potentially important observation is that the proportion of tumors with decreased p120 (45%) was much greater than that of E-cadherin (25%), β-catenin (4%), or α-catenin (17%), indicating that p120 loss may in some cases precede loss of other members of the cadherin–catenin complex. Interestingly, decreased p120 in this study directly correlated with increased PSA levels, an effect not seen for any other member of the cadherin–catenin complex.

The results of these large studies are very similar – both suggest a role for p120 in the progression of prostate adenocarcinoma. The link between decreased p120 and increased PSA levels is particularly interesting from a clinical standpoint, as PSA is currently the marker of choice for early tumor detection. Thus, p120 loss may occur early during tumor progression in the prostate. In both studies the absent and decreased expression of p120 is combined into one category, so the percentage of prostate tumors where p120 is actually lost is unclear.

Lung carcinoma

P120 expression in non-small-cell lung carcinoma has been studied by tissue microarray (Bremnes et al., 2002). Strikingly, p120 loss was observed in 61% of 193 tumor tissue samples, whereas loss or reduction of E-cadherin was detected in only 10%. Thus, p120 loss may precede E-cadherin loss in some cases. Reduced p120 expression correlated with local invasion and with advanced tumor progression (stage, nodal status, and tumor status).

Pancreas, endometrium, melanoma, and kidney

In a study of 20 pancreatic tumors, p120 was altered in 60%. Specifically, p120 was absent in 15%, cytoplasmic with loss of membranous staining in 25%, and heterogeneous in 20% (Karayiannakis et al., 1999). In endometrial adenocarcinomas, p120 abnormalities strongly correlated with poorly differentiated cancer, but were not found in well-differentiated tumors (Miyamoto et al., 2000). The p120 abnormalities observed correlated with changes in E-cadherin and other catenins. In a study of 54 melanomas, p120 was also heterogeneously expressed and frequently absent (Zhang and Hersey, 1999). No differences in p120 immunostaining were seen in cases of sarcomatoid renal cell carcinoma (Kuroiwa et al., 2001), making it one of the few examples of tumors where p120 defects were not observed.

Overall, the abundance of new evidence suggests that p120 is indeed frequently altered or lost in human tumors. Potential mechanism(s) (e.g. gene mutation, promoter methylation, signaling, etc.) have not yet been investigated, but there is now considerable circumstantial evidence that p120 loss may in some cases precede loss of E-cadherin.

Moreover, p120 loss is often associated with tumor grade and stage, indicating a correlation with biological aggressiveness.

Tumor microenvironment and the p120 paradox

The extent of p120 loss evident from the above studies is surprising because p120 is rarely absent from tumor-derived cell lines. In contrast, the frequent loss of E-cadherin in tumor tissue is paralleled by frequent derivation of carcinoma cell lines that lack E-cadherin. Thus, the finding of frequent p120 loss in tumor tissue but not tumor cell lines is paradoxical.

It has been suggested that p120 loss is an artifact of harsh antigen-retrieval procedures. In E-cadherin-deficient cells, p120 is often cytoplasmic and might be removed under such conditions. Diffuse localization of E-cadherin complexes in cancerous tissue might also give rise to staining that under-represents p120 levels, and there are other complicating factors associated with methods of tumor-tissue sampling (e.g. heterogeneity within tumors), and quantification of otherwise qualitative immunohistochemical data. On the other hand, most cytoplasmic proteins are permanently cross-linked in place by formaldehyde fixation and would be expected to persist, even after harsh antigen retrieval methods. Indeed, cytoplasmic p120 was clearly demonstrated in most of the studies cited above. Moreover, there are now over 18 reports of p120 loss in multiple tumor types, an observation that is increasingly difficult to dismiss as an artifact.

One possibility is that p120 absence from cells is tolerated in the context of the tumor microenvironment but not *in vitro*. Thus, signaling derived *in vivo* from tumor stroma or extracellular matrix may circumvent or substitute for a required p120 function. *In vitro*, such signals may be absent, such that cells explanted from p120-deficient tumors fail to survive. Alternatively, the tumor microenvironment *in vivo* might actively suppress p120 expression, and the suppressive mechanism could be lost when the cells are cultured *in vitro*. Thus, explanting p120-deficient tumor tissue to the culture dish might restore p120 expression. The resolution of these issues, and whether p120 expression is modulated by the microenvironment during tumor progression and metastasis, may provide clinically useful information relevant to understanding and managing metastasis.

Tumor suppressor or metastasis promoter

Recent identification and characterization of a unique p120-deficient carcinoma cell line has provided the first molecular clues as to potential consequences of p120

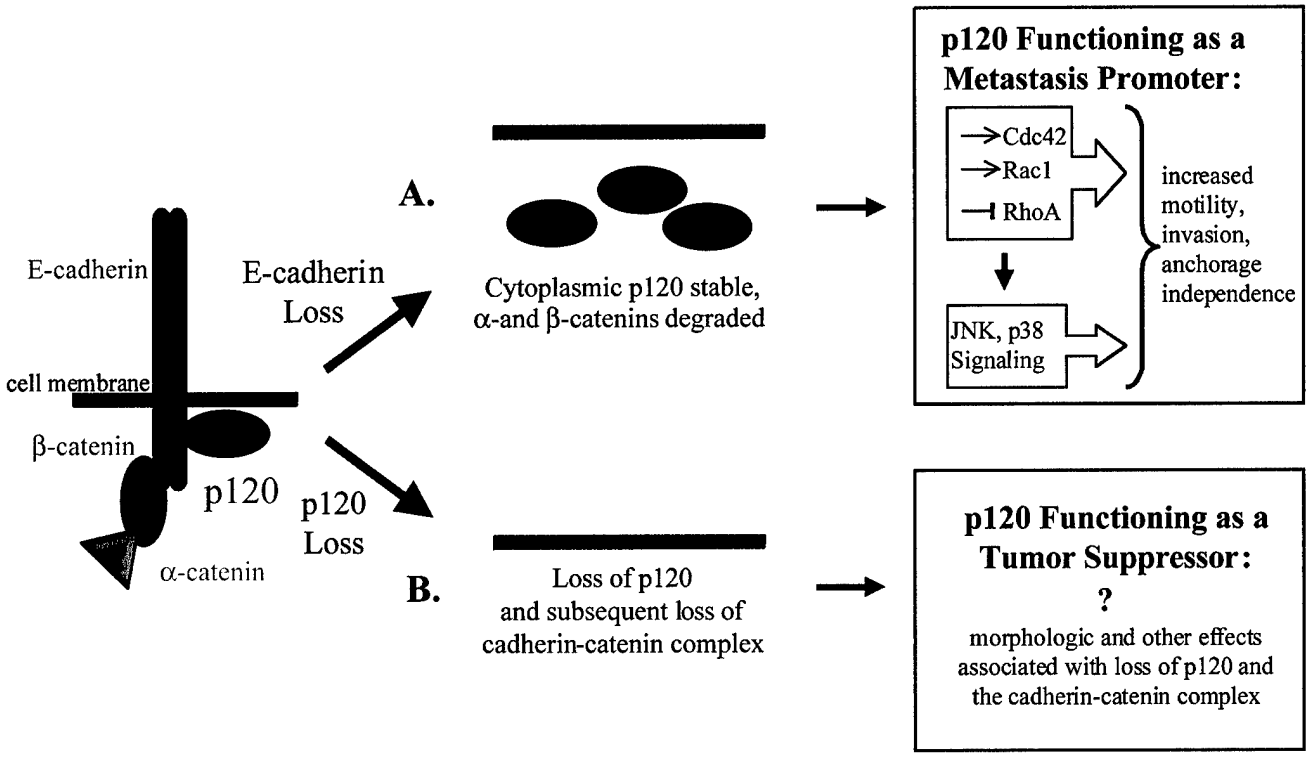

Fig. 1 Hypothetical roles for p120 as metastasis promoter or tumor suppressor. **A** E-cadherin loss precedes p120 loss. E-cadherin loss leads to degradation of α and β-catenins, but p120 remains stranded in the cytoplasm. Loss of adhesion by itself is probably not sufficient to explain the complex behavior of metastatic cells. Instead, the resulting accumulation of p120 in the cytoplasm is postulated to mediate at least some of these effects through regulation of Rho-GTPases, which regulate lamellipodia, filopodia, stress fibers, and many other structures associated with cell motility, morphology, and invasiveness. Rho-GTPases also regulate MAPK (JNK, p38) signaling pathways, which are likely to further modulate aspects of the metastatic program. Thus, p120 may function as a metastasis promoter under these conditions. **B** p120 loss precedes E-cadherin loss. The pathology literature (see Table 1) indicates that p120 is frequently downregulated in tumors. In some cases, p120 loss may be the initial event leading ultimately to inactivation of the cadherin complex. Mechanistic studies suggest that p120 loss destabilizes E-cadherin, which in turn is predicted to reduce levels of α- and β-catenins. The end consequences of this general downregulation of the cadherin complex are uncertain, but likely involve far reaching changes in adhesion, signaling, and morphology, events broadly relevant to tumor progression.

loss in tumors (Ireton et al., 2002). SW48 cells are colon carcinoma cells with mutated p120 genes and sharply reduced levels of p120 protein, providing a rare opportunity to examine the consequences of p120 loss and reconstitution in a tumor-derived cell line. Interestingly, the p120 deficiency appears to result in strongly reduced levels of E-cadherin, which in turn leads to loosely organized cells that fail to maintain epithelial morphology. Restoring p120 rescues the epithelial phenotype, apparently by stabilizing and restoring normal levels of E-cadherin (Ireton et al., 2002). Thus, it is possible that morphologic and behavioral changes in some tumors are due to p120 loss and consequent destabilization of E-cadherin. These data suggest a novel mechanism by which E-cadherin might be downregulated in tumors.

In light of these data, and previously reported models describing a potential function for p120 as a metastasis promoter (Anastasiadis et al., 2000), it is possible that roles for p120 during tumor progression differ, depending on the order in which p120 or E-cadherin are downregulated (Fig. 1). The new data suggest that if p120 is lost first, E-cadherin levels will fall significantly (Ireton et al., 2002), which is likely to be paralleled by reduced levels of α- and β-catenins (Nagafuchi et al., 1991; Papkoff, 1997). Indeed, in studies where all components of the cadherin complex were examined in individual tumors, there is evidence that p120 loss is sometimes associated with general downregulation of all members of the complex. Moreover, several clues in the pathology literature described above are consistent with frequent and early p120 loss in tumors. As E-cadherin is well established as a tumor suppressor, it follows that p120 may function similarly through its ability to stabilize and/or regulate E-cadherin.

On the other hand, in the event that E-cadherin is lost first, p120 may directly and actively promote metastasis. From a mechanistic standpoint, it seems unlikely that E-cadherin loss by itself can fully explain the metastatic phenotype because loss of adhesion does not necessarily translate into increased motility and invasiveness. p120

is thought to modulate the activities of Rho-GTPases (reviewed in Anastasiadis and Reynolds, 2001). These effects are particularly pronounced when p120 is overexpressed, a condition that results in increased levels of p120 in the cytoplasm and significant changes in cell morphology and/or motility (Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000; Reynolds et al., 1996). Upon loss of E-cadherin, p120 translocates from the membrane to the cytoplasm (Thoreson et al., 2000), where it may actively promote some of the exaggerated effects observed upon p120 overexpression. Alternatively, the untethered (cytoplasmic) p120 pool may have increased access to the nucleus where its role has yet to be established.

Together, these observations suggest that p120 may behave as either tumor suppressor or metastasis promoter, depending on the order and context of E-cadherin and p120 loss.

Conclusions

In summary, accumulating evidence indicates that p120 is frequently lost or abnormally expressed in human tumors. Paradoxically, these observations have not been extended to cultured tumor cell lines, with the single exception of the colon carcinoma cell line SW48. In these cells, exogenously expressed p120 stabilizes E-cadherin, thereby restoring epithelial morphology. Together, the tumor pathology and mechanistic data are consistent with a role for p120 as a tumor suppressor. Alternatively, p120 may promote metastasis in tumors where E-cadherin expression is lost first and p120 is retained. A potential explanation for the very low frequency of p120 loss in cultured cell lines is that p120 loss may be permitted in the context of the tumor microenvironment but is incompatible with cell survival *in vitro*. Thus, it will be important in future studies to examine the mechanism of p120 loss, whether this is indeed an early event in the genesis of some tumors, and whether properties associated with the tumor microenvironment can affect p120 expression. It is possible that p120 expression can be restored in tumors by pharmacologic means as a strategy for repressing metastasis.

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Article

A core function for p120-catenin in cadherin turnover

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p120-catenin stabilizes epithelial cadherin (E-cadherin) in SW48 cells, but the mechanism has not been established. Here, we show that p120 acts at the cell surface to control cadherin turnover, thereby regulating cadherin levels. p120 knockdown by siRNA expression resulted in dose-dependent elimination of epithelial, placental, neuronal, and vascular endothelial cadherins, and complete loss of cell–cell adhesion. ARVCF and δ-catenin were functionally redundant, suggesting that proper cadherin-dependent adhesion requires the presence of at least one p120 family member. The data reveal a core function of p120 in cadherin complexes, and strongly predict a dose-dependent loss of E-cadherin in tumors that partially or completely down-regulate p120.

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Introduction

p120-catenin (p120) is the prototypic and most abundant member of an Arm-domain protein subfamily that includes ARVCF, δ-catenin, and p0071 (for review see Anastasiadis and Reynolds, 2000). p120 was originally described as a substrate for Src- and receptor tyrosine kinases (Reynolds et al., 1989, 1992), and later was identified as a catenin (Reynolds et al., 1994; Shibamoto et al., 1995), one of several cofactors that interact with the cadherin tail and modulate cadherin function (for review see Anastasiadis and Reynolds, 2000). The classical catenins, α- and β-catenin, bridge the cadherin cytoplasmic domain to the underlying actin cytoskeleton. p120 is required to stabilize epithelial cadherin (E-cadherin) in SW48 cells (Ireton et al., 2002), and may also regulate cadherin–cytoskeletal connections indirectly through functional interactions with Rho GTPases (Anastasiadis et al., 2000; Noren et al., 2000; Grosheva et al., 2001; Magie et al., 2002; for review see Anastasiadis and Reynolds, 2001), but the underlying mechanisms have not been established.

E-cadherin is the main cell–cell adhesion molecule in epithelial tissues and is regarded as a master organizer of the epithelial phenotype (Takeichi, 1995). Direct mutation of the E-cadherin gene in gastric and lobular breast carcinomas indicates a classical tumor suppressor role in some tumors (Oda et al., 1994; Berx et al., 1995). In late-stage carcinomas of all types, E-cadherin down-regulation occurs frequently via epigenetic mechanisms (Comijn et al., 2001; Matsumura et al., 2001) and is closely correlated with the transition to metastasis (Frixen et al., 1991; Vleminckx et al., 1991; Birchmeier and Behrens, 1994; Perl et al., 1998). Together, these data establish E-cadherin as a tumor and/or metastasis suppressor, depending on the mechanism and timing of E-cadherin down-regulation (for review see Yap, 1998; Nollet et al., 1999).

In the event of E-cadherin down-regulation, α- and β-catenins are rapidly degraded (Nagafuchi et al., 1991) via an adenomatous polyposis coli–dependent mechanism (Polakis, 2000) that ultimately targets β-catenin for destruction by the proteosome (for review see Kikuchi, 2000). In contrast, p120 is stable in the absence of cadherins and becomes stranded in the cytoplasm (Thoreson et al., 2000). Ourselves and others have postulated that cytoplasmic p120 actively drives the metastatic phenotype in cadherin-deficient cells through inappropriate activation/suppression of various Rho-GTPases such as Rac1 and RhoA (Anastasiadis et al., 2000; Noren et al., 2000; Anastasiadis and Reynolds, 2001; Grosheva et al., 2001). These data suggest a metastasis promoter role for p120 when mislocalized through prior loss of E-cadherin.

Down-regulation of p120 occurs frequently in colon, prostate, breast, lung, and other carcinoma types (for review see Thoreson and Reynolds, 2002), but the consequences are unknown. Paradoxically, it is rare to see p120 down-regulation in established tumor cell lines. The lone exception is the SW48 colon carcinoma cell line, where genetic alterations result in extremely low levels of a mutated p120 that lacks the carboxy terminus (Ireton et al., 2002). Restoring normal

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Key words: tumor suppressor; p120-catenin; cell adhesion; tumor progression; metastasis

Abbreviations used in this paper: E-cadherin, epithelial cadherin; h siRNA, human small interfering RNA; HUAEC, human umbilical aortic endothelial cells; m siRNA, murine small interfering RNA; N-cadherin, neuronal cadherin; p120, p120-catenin; P-cadherin, placental cadherin; pRS, pRetroSuper; siRNA, small interfering RNA; VE-cadherin, vascular endothelial cadherin.

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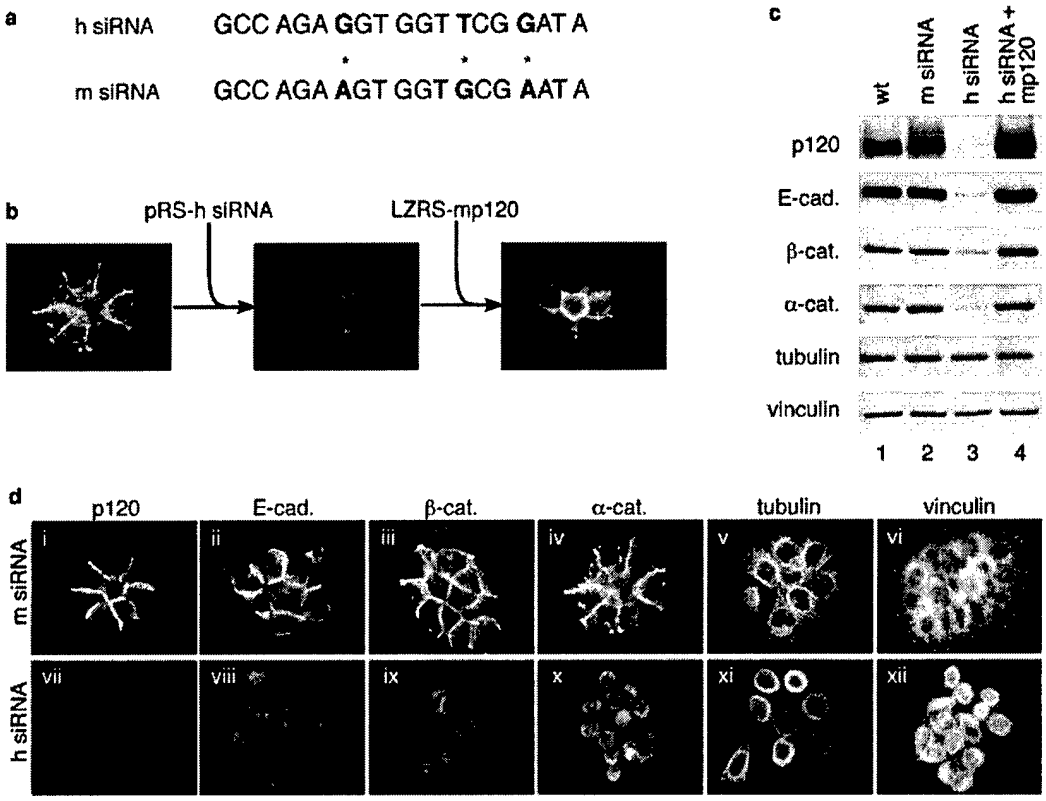

Figure 1. **p120 knockdown eliminates the E-cadherin complex and abolishes adhesion.** (a) Human and murine p120 siRNAs (h siRNA and m siRNA, respectively) were generated against homologous human and murine sequences that contain three mismatches at the nucleotide level (asterisks). (b) Schematic depicting a novel method for in vitro p120 knock-down and knock-up. Human p120 was knocked down using the retroviral vector pRS to express human-specific p120 siRNA, and stable cell lines were selected. p120 was then reexpressed (knock-up) by infecting the knock-down cell line with an LZRS retrovirus containing murine p120 cDNA. (c) Wild-type A431 cells (lane 1) were infected with virus carrying the control m siRNA (lane 2) or h siRNA (lane 3), and stable cell lines were isolated. p120 expression was restored (knock-up) by infecting h siRNA–expressing cells with retrovirus containing murine p120 (lane 4). The indicated cadherin complex proteins were analyzed by Western blotting whole cell lysates. E-cadherin, β-catenin, and α-catenin levels were substantially reduced in p120 knockdown cells, and restoring p120 reversed the effect. (d) p120 (i and vii), E-cadherin (ii and viii), β-catenin (iii and ix), α-catenin (iv and x), tubulin (v and xi), and vinculin (vi and xii) were localized by immunofluorescence in stable A431 cell lines expressing the control m siRNA (i–vi) or h siRNA (vii–xii). Cells were plated sparsely to allow colonies to emerge from single cells. Note that p120 knockdown cells lack cadherin complexes and have lost cell–cell adhesion. The cadherin complex is selectively targeted because the levels of tubulin and vinculin are unaffected.

levels of full-length p120 expression in these poorly organized cells stabilized E-cadherin and caused a striking rescue of epithelial morphology. Thus, in SW48 cells at least, p120 appears to be essential for E-cadherin stability and function (Ireton et al., 2002). On the other hand, recent reports in *Drosophila* (Myster et al., 2003; Pacquelet et al., 2003) and *Caenorhabditis elegans* (Pettitt et al., 2003) indicate that p120 is not essential, and that its absence causes only minor defects that are not fully apparent unless complemented by weak alleles of E-cadherin or α-catenin.

Here, to clarify the role of p120 in mammalian cells, we have knocked down p120 with siRNA in cells expressing epithelial (E-), placental (P-), neuronal (N-), and vascular endothelial (VE-) cadherins. We report that each of these cadherins, as well as α- and β-catenins, were rapidly degraded in the absence of p120, resulting in loss of cell–cell adhesion. The effect was clearly dose dependent, indicating that p120 expression levels may directly determine cadherin levels. Degradation of p120-uncoupled cadherin occurred after its arrival at the surface, indicating that p120 regulates cadherin turnover at the level of internalization or recycling. p120 homologues ARVCF and δ-catenin could substitute for p120, so at least one family member is likely required to maintain adhesion. Thus, cadherin complexes are rapidly turned over and degraded in mammalian cells in the absence of direct interaction with p120 or a p120 family member. These observations establish a core function for p120 in the cadherin complex and have additional implications in support of a role for p120 in tumor suppression.

Results

p120 loss leads to loss of the cadherin complex

To directly address the general consequences of p120 deficiency, we stably expressed p120-specific siRNA using the pRetroSuper (pRS) retrovirus to knockdown p120 in mammalian cell lines (Fig. 1). Human and murine p120 siRNAs (h siRNA and m siRNA, respectively) were generated against homologous human and murine sequences that differ by three mismatches at the nucleotide level (Fig. 1 a). Pi-

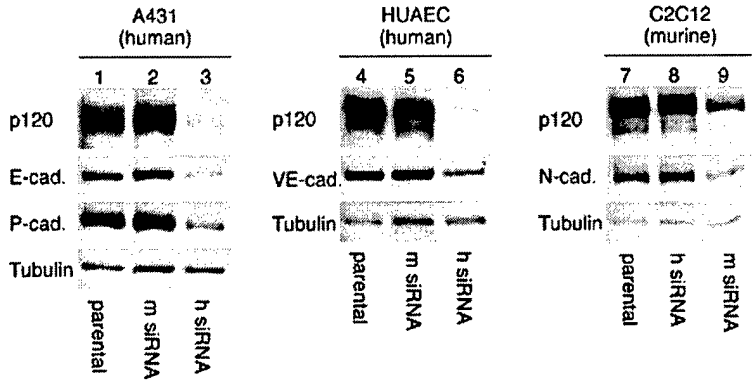

Figure 2. **The p120-associated destruction mechanism is common to multiple cadherins.** A431 (human cervical carcinoma), HUAEC, and C2C12 (mouse myocyte) cells express E- and P-cadherin, VE-cadherin, and N-cadherin, respectively. Each cell line was infected with either human- or murine-specific p120 siRNA retrovirus to generate polyclonal knockdown cell lines, and levels of p120 or E-, P-, VE-, and N-cadherins were assayed by Western blotting of whole-cell lysates. Tubulin levels were used as a loading control. p120 knockdown reduced expression of all these cadherins, indicating that its function is common to most (if not all) p120-associated cadherins. Note that the effects of the h and m siRNAs used for knockdown and control in the human cell lines are reversed in the murine cell line C2C12.

lot experiments revealed that the h siRNA strongly knocked down p120 levels in human cells, but not murine cells, and vice versa. E-cadherin levels were also severely reduced by p120 knockdown in several different epithelial cell lines. These data indicate that the stabilizing effect of p120 is not limited to SW48 cells, but represents a mechanism that is likely common to all E-cadherin–expressing cells.

By intentionally targeting the above siRNA oligos to human and murine sequences that differed by several nucleotides, it was relatively straightforward to efficiently "knock down" p120 with the human-directed siRNA (pRS-h siRNA) and subsequently "knock up" p120 by infection with pLZRS-mp120, a retrovirus containing the murine p120 cDNA (Fig. 1 b). Restoring p120 levels by expressing murine p120 reversed the effects of the h siRNA and restored adhesion (Fig. 1, b and c). It is worth noting that this method is generally applicable to any protein. If a homologous gene is not available, a knock-up construct can be generated by making silent mutations in the region targeted by the siRNA. The method is a simple in vitro equivalent of transgenic knock-out and knock-in technology, and essentially solves the common dilemma associated with expressing mutant proteins in cells that already contain high levels of an endogenous counterpart. To our knowledge, this is the first example of this broadly applicable method.

To examine the effects of p120 knockdown in detail, we isolated stable clones of A431 cells expressing p120-specific siRNA and characterized them by Western blotting (Fig. 1 c) and by immunofluorescence (Fig. 1 d). p120 was nearly eliminated by h siRNA (Fig. 1 c, lane 3), but not by m siRNA (Fig. 1 C, lane 2), and p120 loss induced near complete loss of E-cadherin. Levels of α- and β-catenin were also severely reduced, as expected from the fact that these catenins are stabilized via interaction with cadherins. Thus, p120 loss essentially eliminated the entire cadherin complex. Levels of vinculin, which concentrate at focal adhesions in these cells, were unaffected, as were levels of tubulin.

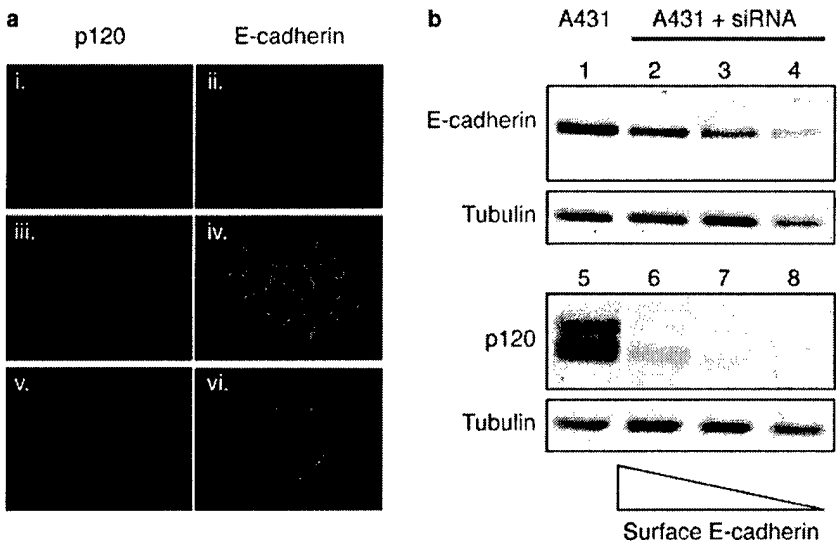

Figure 3. **p120 levels act as a set point mechanism for determining cadherin levels.** (a) Assay of relationship between p120 and E-cadherin levels by immunofluorescent staining. A431 cells expressing p120 siRNA were infected with the murine p120 retrovirus and plated sparsely so that individual clones could emerge that expressed widely varying amounts of murine p120. Cells were co-stained by immunofluorescence to examine the p120–E-cadherin relationship and its affect on cell–cell adhesion. p120 loss (i) caused complete loss of E-cadherin (ii) and the cells were nonadhesive. Intermediate levels of p120 expression (panel iii) permitted intermediate levels of E-cadherin (panel iv), and cell–cell adhesion was partially restored. Higher than normal levels of p120 (panel v) strongly induced E-cadherin (vi) and cell–cell adhesion was robust. These experiments reveal a direct relationship between p120 and E-cadherin levels, and the extent of cell–cell adhesion is directly affected. (b) Quantitative assessment of relationship between p120 and E-cadherin levels. A polyclonal population of cells expressing p120 siRNA was generated by retroviral infection. Individual clones within the population express different levels of p120 depending on integration events that affect the efficiency of the siRNA expression. Using E-cadherin antibodies (HECD-1), the cells were separated by FACS® into pools expressing progressively lower levels of E-cadherin. Cell lysates from the samples were split and then Western blotted with anti-p120 (mAb pp120) or anti-E-cadherin (C-20820).

Analysis of the p120 knockdown cells by immunofluorescence revealed near complete loss of junctional E-cadherin, loss of α- and β-catenins, and loss of cell–cell adhesion (Fig. 1 d). It is noteworthy that other adhesion systems (e.g., desmosomes) cannot compensate for loss of the core components of the adherens junction. These observations reveal that p120 is essential for adhesion and suggest a core function for p120 in regulating cadherin turnover.

The requirement for p120 is common to other cadherins

To determine whether the consequence of p120 knockdown pertains only to E-cadherin, we repeated the experiments described in Fig. 1 on cells expressing E-, P-, VE-, and N-cadherins (Fig. 2). A431 (human cervical carcinoma), human umbilical aortic vascular endothelial (HUAEC), and C2C12 (murine myoblast) cells were selected because they express E- and P-cadherin, VE-cadherin, and N-cadherin, respectively. Interestingly, the levels of each of these cadherins were substantially reduced by p120 knockdown (Fig. 2, lanes 3, 6, and 9). Note that because C2C12 cells are murine, the constructs are reversed relative to the human lines; m siRNA is the knockdown construct and the h siRNA is the control. The knockdown levels in these experiments are not quite as striking as in the clonal cell lines represented in Fig. 1 because they are polyclonal cell lines, and therefore represent the average siRNA expression and knockdown from multiple integration events. Nonetheless, these data indicate clearly that the mechanism of stabilization by p120 is common to a wide variety of cadherins, probably all cadherins that bind p120.

p120 levels directly determine cadherin levels

To more accurately quantify the relationship between p120 and cadherin expression, we infected A431 cells with the p120 siRNA virus and analyzed individual cell clones by coimmunofluorescence for p120 and E-cadherin (unpublished data). We also performed the reverse experiment (knock-up) by introducing murine p120 into the h siRNA-expressing A431 cells (Fig. 3). In all cases, there was a striking correlation between the levels of p120 and E-cadherin, which was also reflected by the extent of cell–cell adhesion. In the absence of p120, there was essentially no E-cadherin present (Fig. 3, i and ii). By contrast, intermediate levels of p120 caused intermediate levels of E-cadherin and partial restoration of epithelial morphology (Fig. 3, iii and iv). When murine p120 was expressed at higher than normal levels, E-cadherin levels were correspondingly elevated and exceeded the wild-type levels observed in the parental cell lines (Fig. 3, v and vi; see also Fig. 1 c). Panels v and vi are overexposed because the common exposure time for the entire panel was chosen to allow better visualization of the low and intermediate p120 levels.

We also quantified the relationship between p120 and E-cadherin expression by FACS® analysis of a population of p120 siRNA-infected cells with mAb-HECD1, which recognizes the extracellular domain of human E-cadherin (Fig. 3 b). The cells were sorted into pools with progressively decreasing levels of surface E-cadherin. Cell lysates were generated from each pool, divided in half, and then Western blotted for E-cadherin and p120 (Fig. 3 b). As in the immunofluorescent assays, the levels of p120 closely paralleled the levels of E-cadherin.

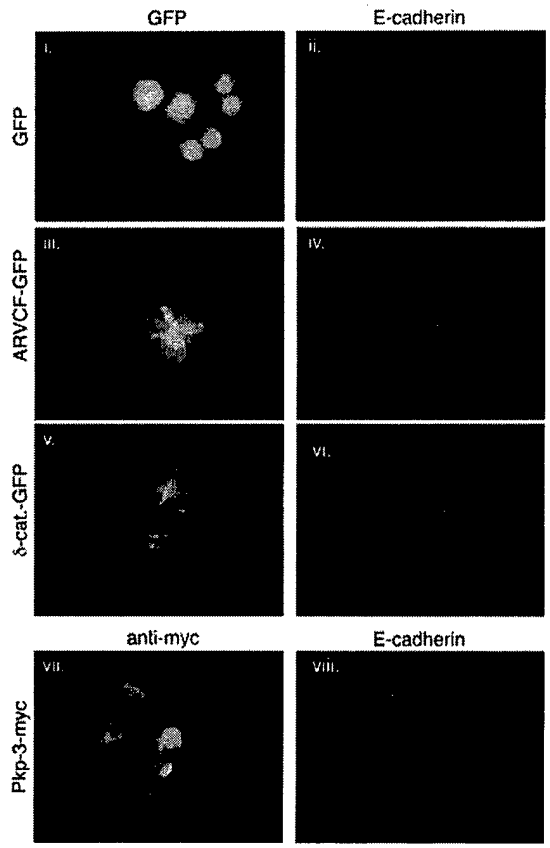

Figure 4. **Redundant roles for p120 family members ARVCF and δ-catenin.** A431 cells stably expressing human p120 siRNA were transiently transfected with ARVCF-GFP, δ-catenin–GFP, or myc-tagged plakophilin-3 (Pkp-3-myc). 24 h after transfection, cells were plated sparsely and individual colonies grew for 2 d. Levels of the transfected proteins and E-cadherin were then analyzed by immunofluorescence. GFP expression alone (i, eluminated cells) did not affect E-cadherin levels (ii). Both ARVCF-GFP (iii) and δ-catenin–GFP (v) substantially increased levels of E-cadherin (iv and vi) and rescued cell–cell adhesion. In contrast, plakophilin-3 (vii), a p120-related protein that does not bind classical cadherins, but had no effect on E-cadherin levels (viii) or cell–cell adhesion.

Together, these data show that E-cadherin levels faithfully reflect the level of p120 expression in individual cells, and show that the levels of E-cadherin can be experimentally titrated by increasing or decreasing the levels of p120.

p120 family members can functionally substitute for p120

In most epithelial cell lines, p120 is abundant and its close relatives such as ARVCF and δ-catenin are poorly expressed or absent. Although p120 knockdown was sufficient to nearly eliminate E-cadherin in several epithelial cell lines tested, the effect was incomplete in cells such as the colon carcinoma cell line HCT116. An obvious explanation is that p120 family members might partially or completely substitute for p120, depending on their relative abundance. Indeed, HCT116 cells are unusual in that they express moderate levels of ARVCF (unpublished data). To determine whether other p120 relatives can also regulate E-cadherin

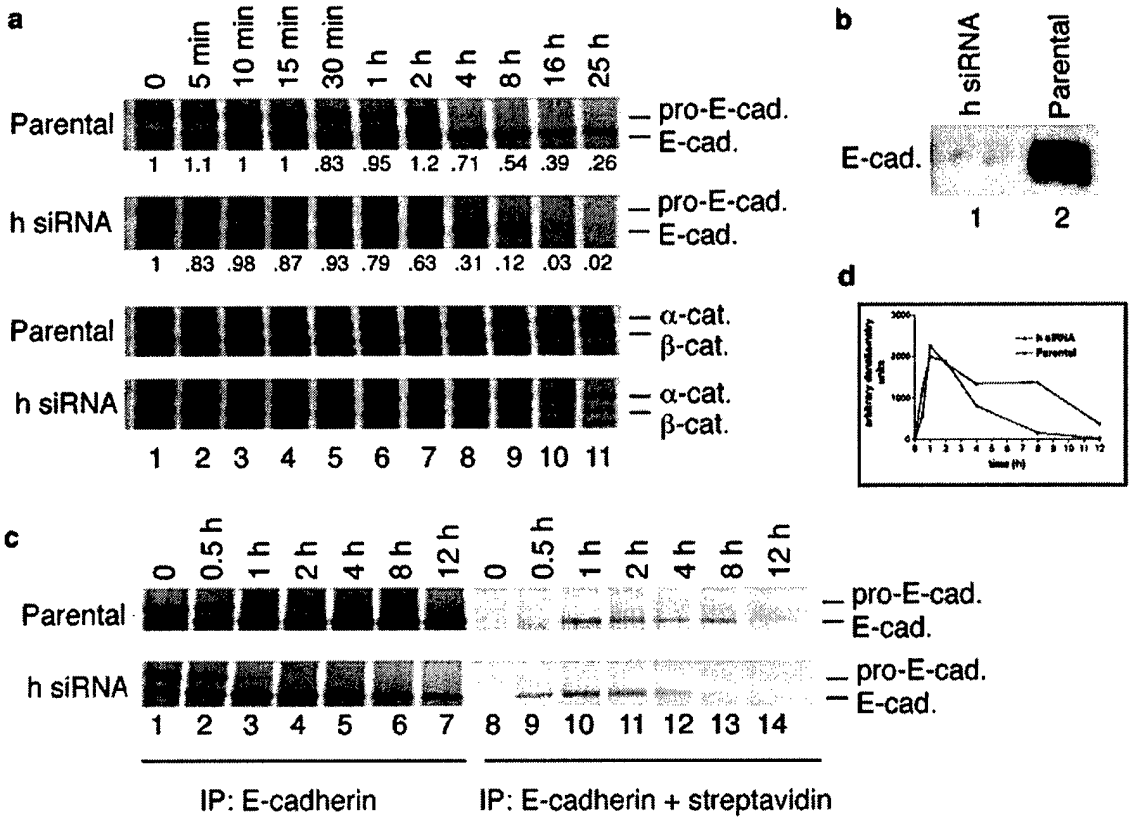

Figure 5. **p120 regulates E-cadherin turnover at the cell membrane.** (a) E-cadherin synthesis and processing in p120 knockdown cells. E-cadherin turnover was examined by pulse-chase analysis of parental and h p120 siRNA-expressing A431 cells. α- and β-Catenin processing from the same experiment are shown below. Chase times are indicated across the top. At chase time 0 (15 min after initiation of the pulse labeling), E-cadherin synthesis was identical in the presence and absence of p120 (a, compare E-cadherin bands). The processing of the pro- (pro-E-cad) and mature (E-cad.) forms were identical for at least 1 h. Soon thereafter, E-cadherin degradation was significantly accelerated in the absence of p120. (b) Analysis of total E-cadherin surface levels in parental and p120 knockdown A431 cells. The parental and p120 knockdown A431 cells were biotinylated for 20 min at 4°C to label surface cadherins. To specifically measure the surface levels, E-cadherin was first immunoprecipitated directly with E-cadherin mAb HECD-1. The sample was eluted with 0.5% SDS and then reprecipitated with streptavidin-coated beads to isolate the surface-labeled pool. E-cadherin levels at the surface in p120 knockdown cells (lane 1) are at least 100-fold diminished relative to the parental cells (lane 2). The result in lane 2 shows that the surface E-cadherin can be efficiently labeled (and detected) by this method. (c) Tracking the arrival of newly synthesized E-cadherin to the cell surface. The methods in a and b were combined to determine whether newly synthesized E-cadherin could transit to the cell surface in the absence of p120. The results in c were quantified by densitometry and represented graphically in d. Parental and p120 knockdown (h siRNA) cells were labeled with [^{35}S]methionine for 15 min, chased at 37°C for the times indicated across top, and placed on ice (4°C) to suspend trafficking. Cell surface proteins were immediately biotinylated at 4°C for 20 min as in b. Surface E-cadherin was then isolated as in b, and the nascent [^{35}S]methionine E-cadherin pool was visualized by SDS-PAGE and radiography. Nascent E-cadherin appeared at the surface at 30 min and peaked at 1 h. The absence of p120 had no effect on this result. Therefore, p120 is not required for E-cadherin synthesis or trafficking, but is essential to regulate E-cadherin turnover soon after its arrival at the cell surface.

turnover, we transiently expressed GFP-labeled ARCVF or δ-catenin in A431 cells that lack p120 as a result of siRNA knockdown (Fig. 4). As a negative control, we also tested plakophilin-3, a more distant p120 relative that binds desmosomal (but not classical) cadherins. A431 cells expressing p120 siRNA alone were almost completely E-cadherin negative (Fig. 4, i and ii), as described earlier in this paper, and were not affected by GFP expression (Fig. 4 i, fluorescent cells). ARVCF (Fig. 4, iii) and δ-catenin (Fig. 4, v) localized to adherens junctions and efficiently rescued adhesion by restoring normal E-cadherin levels (Fig. 4, iv and vi). In contrast, myc-tagged plakophilin-3 (Fig. 4 vii, stained cells) did not affect cadherin levels (Fig. 4 viii) and failed to restore cell–cell contacts. Thus, there is a clear redundant role among close family members with regard to cadherin stabilization, and the occasional significant presence of a p120 family member (e.g., ARVCF in colon HCT116 cells) is likely to account for the fact that E-cadherin loss does not perfectly parallel p120 loss in some cell lines.

Mechanism of E-cadherin loss

p120 reportedly is the first of the catenins to bind newly synthesized N-cadherin, and coprecipitates with the nascent precursor form of N-cadherin (Wahl et al., 2003). Because of the extraordinary efficiency of E-cadherin destruction after p120 knockdown, we first considered the possibility that p120 binding was necessary to stabilize E-cadherin during or after protein translation and before arrival at the cell surface.

To examine E-cadherin synthesis in the absence of p120, we labeled the p120 knockdown A431 cells with [^{35}S]methionine and performed pulse-chase experiments (Fig. 5 a). Interestingly, the rate of E-cadherin synthesis was unaffected by the absence of p120 (Fig. 5 a, compare top panels). Moreover, the processing and turnover of both the precursor and mature forms of E-cadherin were identical for at least 1 h after the pulse, after which the cadherin degradation curves diverged rapidly. Degradation of α- and β-catenins paralleled the loss of E-cadherin, as expected from the fact that these catenins are stabilized by cadherin binding.

The fact that the newly synthesized cadherin behaved identically in the presence and absence of p120 for 1 h, and until after the precursor form disappeared, suggests that cadherin degradation occurred after arrival at the cell surface. The result was initially surprising because examination of total surface levels of E-cadherin in the p120 knockdown cells (h siRNA) and parental cell lines (Fig. 5 b) showed that although surface E-cadherin could be efficiently isolated by biotin labeling and streptavidin pulldown (e.g., Fig. 5 b, lane 2), it was ~100-fold less abundant in the p120-deficient cells (Fig. 5 b, compare lane 1 with lane 2).

To definitively address this issue, we combined the pulse-chase and biotin surface-labeling strategies in order to selectively examine the fate of the nascent E-cadherin molecules with respect to their arrival at the cell surface (Fig. 5 c). The pulse labeling was conducted as in Fig. 5 a, except that surface E-cadherin was subsequently biotin labeled (as in Fig. 5 b) at each time point after the pulse chase. The surface-labeled cadherins were then isolated by streptavidin pulldown, and nascent cadherins were visualized by SDS-PAGE and autoradiography. The data show that the rate of nascent E-cadherin arrival at the cell surface is almost identical in the presence and absence of p120 (Fig. 5, c and d; compare parental and siRNA cell lines). The appearance and removal of E-cadherin from the cell surface (Fig. 5 c; E-cadherin + streptavidin immunoprecipitations) are quantified by densitometry and displayed graphically in Fig. 5 d. Note that peak levels of nascent (^{35}S-labeled) E-cadherin at the cell surface occurred at 1 h, and by 4 h, the nascent cadherin was either moving off the surface or getting degraded. The timing is consistent with the 4-h time point in Fig. 5 a, which marks the first interval where degradation of the unbound cadherin sharply accelerates. Clearly, E-cadherin transits normally to the surface in the absence of p120, but is then rapidly turned over.

To identify the mechanism of degradation, we treated p120 knockdown cells with over 30 agents known to inhibit factors that have been reported to affect cadherin stability and turnover. Examples include inhibitors of presenilin-1, caspases, metalloproteinases, and calpain. Cells were incubated for 24 h with predetermined amounts of the various inhibitors, and then analyzed by immunofluorescence (unpublished data) or Western blotting for changes in levels of E-cadherin (Fig. 6). Although the majority of the inhibitors had no affect, several proteosome inhibitors (i.e., PS341, lactacystin, and MG132) significantly blocked E-cadherin degradation (Fig. 6; lactacystin, lanes 1 and 2; PS341, lanes 3 and 4). The reduced amount of E-cadherin at the higher PS341 dose (Fig. 6, compare lane 3 with lane 4) reflects toxicity of this compound. Of the two commonly used lysosomal inhibitors we tried, ammonium chloride (Fig. 6, lane 5; NH_4Cl) had no effect, but chloroquine (Fig. 6, lane 6; Chl) blocked E-cadherin degradation almost as effectively as the proteosome inhibitors. In both the PS341- and chloroquine-treated cells, cytoplasmic pools of E-cadherin increased, but the increased levels were not reflected by increased adhesion or higher surface cadherin levels. Thus, these inhibitors appear to block cadherin degradation, but do not affect internalization. The data suggest that when newly synthesized E-cadherin arrives at the cell surface, p120 is required to prevent the immediate targeting of unbound E-cadherin for degradation by the proteosome and/or lysosome. We conclude that p120 regulates cadherin turnover by controlling either internalization, or possibly an immediately subsequent decision whereby internalized cadherins are sorted into recycling or degradation pathways.

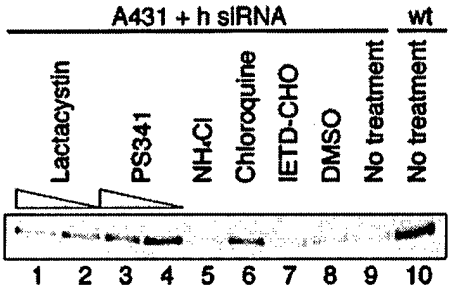

Figure 6. **Mechanism of E-cadherin degradation.** The effects of various inhibitors known to influence cadherin stability were assayed in the stable p120 knockdown A431 cells. E-cadherin levels from samples treated for 24 h (lanes 1–9) were monitored by Western blotting whole-cell lysates and were compared with normal E-cadherin levels in the parental cell line (lane 10). Inhibitor concentrations were as follows: lactacystin 10 μM (lane 1), 3.3 μM (lane 2); PS341 100 nM (lane 3), 33 nM (lane 4), ammonium chloride 5 mM (lane 5), Chloroquine 33 μM (lane 6), and IETD-CHO 10 nM (lane 7). DMSO is the control condition (lane 8). The proteosome inhibitors lactacystin and PS341 increased E-cadherin levels (compare lanes 1–4 to lanes 8 and 9). The lower cadherin levels after 100 nM PS341 (lane 3) relative to the 33-nM treatment (lane 4) is due to toxicity at the higher concentration. Of the lysosomal inhibitors, chloroquine (lane 6), but not ammonium chloride (lane 5), increased E-cadherin levels. A caspase 8 inhibitor (lane 7) that has been shown to inhibit E-cadherin degradation in myeloma cells had no effect.

Discussion

Here, we provide evidence that the core function of p120 in cadherin complexes is to regulate cadherin turnover. Previously, we showed that the stabilizing effect of p120 on E-cadherin in a p120-deficient SW48 cell line involved a post-transcriptional mechanism and required direct p120–E-cadherin interaction (Ireton et al., 2002). However, it was not clear whether this phenomenon was generally applicable beyond SW48 cells, nor could we determine the underlying mechanism. Here, using siRNA and/or p120 reconstitution, we show that E-cadherin levels depend absolutely on p120 expression. Importantly, this set point mechanism is common to other (probably all) p120-binding cadherins because p120 knockdown also induced significant down-regulation

of P-, VE-, and N-cadherins. The timing and location of p120 action argue strongly that p120 regulates adhesion via controlling cadherin turnover at the cell surface. These observations have crucial implications for roles of p120 in cadherin function and cancer.

We believe that the only exception to the requirement for p120 occurs in cells that express p120 family members such as ARVCF or δ-catenin. This qualifier is based in part on cell lines such as HCT116 where the observed reduction in E-cadherin levels after p120 siRNA expression did not perfectly parallel the extent of p120 loss. Indeed, although ARVCF is typically difficult to detect in many epithelial cell lines, it is expressed at moderate levels in HCT116 cells (unpublished data). Our data show that ARVCF and δ-catenin efficiently compensate for p120 loss when ectopically expressed in A431 cell lines expressing p120 siRNA. Despite significant structural and sequence similarity, plakophilin-3 had no effect, presumably because it does not bind classical cadherins. These data strongly imply that surface cadherin stability is invariably dependent on the binding of either p120 or a closely related family member, and the presence of variable levels of p120 family members likely accounts for the discrepancy in cell lines where p120 knockdown does not cause a corresponding loss of resident classical cadherins.

The fact that p120 availability limits cadherin levels has several crucial implications. For example, overexpression of dominant-negative cadherins frequently down-regulates expression of endogenous cadherins (Kintner, 1992; Fujimori and Takeichi, 1993; Zhu and Watt, 1996), but the mechanism is unknown. Our data strongly suggest that a key action of dominant-negative cadherins is the sequestering of endogenous p120, thereby driving the turnover and degradation of endogenous cadherins. In addition, cadherin levels in cells may ultimately be controlled by factors that regulate p120 levels, and competition for interaction with p120 is likely to be physiologically relevant in cells that express more than one cadherin.

In theory, the absence of cadherins in p120-deficient cells indicates either a failure to normally synthesize cadherins or an efficient means of eliminating them when p120 is not present. However, our pulse-chase data indicate that p120 is not required for normal synthesis or transit of cadherin to the cell surface. Instead, p120 absence dramatically accelerates cadherin degradation after its arrival at the surface, indicating a role in regulating cadherin turnover at the membrane (modeled in Fig. 7). Our data do not precisely distinguish the point at which p120 acts to prevent degradation. The simplest explanation is that p120 limits degradation by regulating internalization. Only cadherin-bound p120 is phosphorylated (Thoreson et al., 2000), and p120 phosphorylation is the most likely means of regulating p120–cadherin affinity and/or p120 activity in the complex. We cannot rule out the less likely possibility that once internalized, p120 might control the next step, which targets the endocytosed cadherin for either degradation or recycling back to the surface. Regardless, it is likely that the ultimate destruction of the cadherin in p120-deficient A431 cells resides mainly in the proteosome, and to some extent in the lysosome.

Under normal circumstances, cadherin turnover is constitutive and endocytosis is a crucial mechanism for down-regulating cadherin adhesiveness (Le et al., 1999, 2002; Xiao et al., 2003). Previously, we postulated that p120 acts as a switch, inducing the assembly or disassembly of cadherin complexes through transient signaling events (probably tyrosine and serine phosphorylation), which in turn might regulate cadherin clustering. Our new data strongly favor a mechanism whereby dynamic assembly and disassembly of cadherin complexes is driven primarily by regulation of cadherin turnover rather than physical clustering (Fig. 7). A plausible explanation is that the rate of cadherin turnover is dictated by events at the cell surface that transiently increase or decrease p120 affinity for cadherins. The off state favors internalization/degradation, whereas the on state favors retention/recycling. The low affinity of p120 for cadherins, as judged by coimmunoprecipitation experiments (Thoreson et al., 2000), probably reflects the ability of p120 to rapidly alternate between cadherin-bound and -unbound states. It is worth noting that α- and β-catenins are largely passive play-

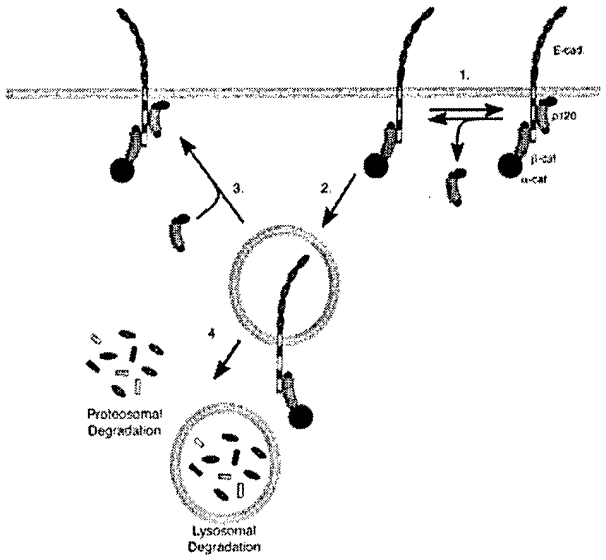

Figure 7. **Model for p120 function in regulating cadherin turnover.** The low affinity of p120 for cadherins, as judged by coimmunoprecipitation experiments, probably reflects the ability of p120 to rapidly alternate between cadherin-bound and -unbound states. (1) Our data suggest that the rate of cadherin turnover is controlled by cell surface events that transiently increase or decrease p120 affinity for cadherins. Thus, cadherin complexes exist in a dynamic equilibrium between p120-bound and -unbound states, which in turn may be regulated by p120 phosphorylation (not depicted). (2) Unbound cadherin is targeted for internalization, possibly via a Hakai-like ubiquitination mechanism (see Discussion). (3) We cannot yet rule out an alternative pathway where p120 binding is irrelevant for internalization, but mediates a sorting decision that recycles internalized cadherin back to the membrane. (4) Regardless of the exact decision point, unbound cadherin is targeted for degradation by the proteosome and/or lysosome. Considerable evidence indicates that signaling events at the cell surface modulate phosphorylation of the cadherin-bound pool of p120. The simplest interpretation of these observations is that p120 phosphorylation regulates its steady-state affinity for cadherins, which in turn regulates adhesion by controlling the rate of cadherin turnover. Note that α- and β-catenin are passive players in this model. They likely participate in clustering and certainly mediate the cytoskeletal interaction (not depicted), but their role may be secondary to regulating surface cadherin levels, which is almost completely determined by p120.

ers in this model. Because their stability is controlled by cadherin binding, their fate is ultimately tied to cadherin levels, which are clearly controlled by p120. Of course, turnover and clustering are not mutually exclusive mechanisms, but our current data suggest that turnover may take precedence.

Recent experiments in *C. elegans* and *Drosophila* indicate that p120 is not essential in these organisms. Indeed, both worms (Pettitt et al., 2003) and flies (Myster et al., 2003) are viable when p120 is removed, and p120-uncoupled E-cadherin can substitute effectively for wild-type E-cadherin in flies (Pacquelet et al., 2003). In contrast, the murine p120 knockout is embryonic lethal (unpublished data). Additionally, our current data show clearly that p120 is essential in mammalian cells. It is possible that mammalian p120 has evolved both additional family members and increased complexity to accommodate the developmental demands of higher organisms.

An unanswered question is the exact targeting mechanism for internalization and/or degradation of cadherins not associated with p120. Because direct binding of p120 to E-cadherin is required, it is possible that p120 binding blocks the interaction of an unknown binding partner (or event) that targets E-cadherin for degradation. Candidates include presenilin-1 (Baki et al., 2001; Marambaud et al., 2002) and Hakai (Fujita et al., 2002), which are reported to compete with p120 for binding the cadherin juxtamembrane domain. Presenilin-1 binding promotes proteolytic degradation of E-cadherin (Baki et al., 2001; Marambaud et al., 2002), whereas Hakai is a ubiquitin ligase that binds tyrosine-phosphorylated E-cadherin, leading to its ubiquitination and destruction (Fujita et al., 2002). Several tyrosine kinase receptors are turned over via a similar mechanism involving the oncogene and ubiquitin ligase Cbl, which binds tyrosine-phosphorylated residues via its classical SH2 domain (for review see Hicke, 1999). However, we were unable to block E-cadherin destruction in the p120 siRNA cell lines with either presenilin or tyrosine kinase inhibitors (unpublished data). Moreover, the mechanism we describe is common to several cadherins, whereas the Hakai mechanism appears specific for E-cadherin. Nonetheless, our data favor a model where an E-cadherin–targeting event is triggered by the absence or transient off-loading of p120.

Finally, several lines of evidence suggest that this new role for p120 in regulating cadherin turnover may be important in cancer. In cell lines, E-cadherin loss leaves p120 stranded in the cytoplasm, but has little effect on p120 levels. It is well established that E-cadherin loss occurs frequently by mutation (Berx et al., 1998) and by epigenetic mechanisms (Comijn et al., 2001; Matsumura et al., 2001) that probably do not involve p120. In contrast, p120 loss clearly represents a different scenario that directly induces loss of E-cadherin, and thus ultimately, the entire cadherin complex. It follows that p120 loss may precede cadherin loss in the reported subset of tumors that have been shown to lack both proteins (for review see Thoreson and Reynolds, 2002). Accumulating evidence suggests that p120 down-regulation occurs frequently in colon, prostate, lung, bladder, breast, and several other malignancies (for review see Thoreson and Reynolds, 2002). p120 is both mutated and underexpressed in the colon carcinoma cell line SW48, and indeed, E-cadherin is indeed strongly down-regulated in these cells, providing the first physiologically relevant example of this phenomena in a carcinoma cell line. However, no other p120-deficient cell lines have been described, and physical alterations in the p120 gene locus have not been associated with malignancy. Together, these observations suggest that p120 down-regulation in tumors occurs by an epigenetic mechanism that has yet to be identified, and raise the possibility that like E-cadherin, p120 acts as a tumor suppressor.

In conclusion, we show that p120 levels determine steady-state levels of functional cadherins by regulating cadherin turnover at the cell surface. This is likely the core function of p120 in the cadherin complex and suggests that cadherin adhesiveness is modulated, in part, by signaling events that dynamically influence p120–cadherin affinity. In addition, p120 is clearly at the top of the cadherin food chain in terms of who controls the overall fate of the complex. Together with reports of p120 down-regulation in a wide range of epithelial tumors, these data suggest a role for p120 as a tumor suppressor.

Materials and methods

Cell culture, infections, and transfections

HUAECs (CC-2535; Cambrex) were thawed at passage one. They were grown in endothelial basal medium (CC-3121; Cambrex) supplemented with EGM SingleQuots® supplements and growth factors (CC-4133; Cambrex). Just before use, HUAEC culture dishes were treated with 0.2% gelatin (Sigma-Aldrich, G1393) in PBS for 20 min at 37°C. Culture conditions for Phoenix cells have been described previously (Ireton et al., 2002), and all other cell lines were cultured as described elsewhere (Anastasiadis et al., 2000). For siRNA expression, cells were infected with pRS and selected with 3 to 5 μg/ml puromycin. As indicated, some cells were infected again with LZRS–mp120–neomycin and selected with 600 μg/ml neomycin. pRS and LZRS retroviruses were produced in the Phoenix cell packaging line as described previously (Ireton et al., 2002). Clonal A431 cell lines were subcloned by limiting dilution. p120 expression was assessed by immunofluorescence and Western blotting. Transient transfections were performed with LipofectAMINE™ 2000 (Invitrogen) according to the manufacturer's instructions.

Immunofluorescence and FACS®

Cells were plated sparsely on glass coverslips and incubated for 2 d before immunofluorescent labeling. Cells were washed once with PBS, then fixed in 3% PFA for 30 min. Fixed cells were washed with PBS/10 mM glycine twice and permeabilized in 0.2% Triton X-100/PBS for 5 min. Cells were again washed in PBS/10 mM glycine and blocked in 3% milk/PBS before staining. Primary antibodies mAb pp120 (Transduction Laboratories), anti-β-catenin C-2206 (Sigma-Aldrich), anti-α-catenin C-2081 (Sigma-Aldrich), anti-E-cadherin C-20820 (Transduction Laboratories), and HECD-1 (a gift from Masatoshi Takeichi, Kyoto University, Kyoto, Japan) were used as described previously (Ireton et al., 2002). Other primary antibodies were used as follows: anti-tubulin (DM1a; Sigma-Aldrich) 1:1000, anti-vinculin (hvin-1; Sigma-Aldrich) 1:400, anti-myc (mAb 9E10) 1μg/ml, and SHE78–7 anti E-cadherin (Zymed Laboratories) 1μg/ml. Secondary antibodies goat anti–mouse IgG1 and IgG2a conjugated either to Alexa® 594 or 488 were used at 1.7 μg/ml. Cells were mounted in ProLong Antifade (Molecular Probes, Inc.) according to the manufacturer's instructions and were visualized on a microscope (Axioplan 2; Carl Zeiss MicroImaging, Inc.) with Immersol 518F oil (Carl Zeiss MicroImaging, Inc.) using a 63× Plan Apochromat 1.4 aperture objective lens (Carl Zeiss MicroImaging, Inc.). Pictures were acquired using a camera (Orca-ER; Hamamatsu) and Openlab v3.1.4 software (Improvision).

To isolate pools of cells expressing different levels of E-cadherin, a p120 siRNA-infected A431 cell population was sorted by FACS® as follows: cells were dissociated with GIBCO BRL cell dissociation buffer (enzyme free, PBS based) at 37°C for 45 min. Single-cell suspensions were enhanced by repeated pipetting, washed in PBS containing 1% serum, and then labeled with E-cadherin mAb HECD1 (10 μg for 5×10^6 cells in 1 ml), followed by washing and then additional labeling with the secondary antibody Alexa® 488–conjugated goat anti–mouse IgG (1/1,000 dilution in 1 ml; Molecular

Probes, Inc.). After washing, cells were labeled with 7AAD (Molecular Probes, Inc.) to discriminate dead cells, and subjected to FACS® using a FACStar PLUS™ cell sorter (Becton Dickinson). All procedures were performed at 4°C to prevent E-cadherin endocytosis. Four gates were set based on preliminary experiments designed to separate cells into four categories of cells expressing high to low levels of E-cadherin. The resulting pools were expanded and then analyzed by Western blotting for p120 and E-cadherin levels.

Pulse chase, biotinylation, and cell surface trafficking

Pulse-chase experiments were performed exactly as described previously (Ireton et al., 2002). Biotinylation and the rate of cell surface trafficking were also performed exactly as described previously (Bonifacino et al., 2003). In brief, cells were plated at 5×10^5 cells per 60-mm dish for 36 h before pulse chase. Cells were ^{35}S-labeled for 15 min before chase. At the end of the chase, cell surface proteins were labeled with 1 mg/ml EZ-Link sulfo-NHS-SS-biotin (Pierce Chemical Co.) at 4°C for 30 min. E-cadherin was immunoprecipitated from NP-40 cell lysates, and surface cadherin was detected by dividing the E-cadherin immunoprecipitations in half, eluting E-cadherin from the beads with 0.5% SDS, reconstituting elutions in 50 mM Tris-HCl (pH 7.5), and pulling down biotinylated E-cadherin with 10 μl per sample of packed streptavidin-coated agarose beads (Sigma-Aldrich) for 1 h at 4°C. Samples were washed three times with 50 mM Tris-HCl (pH 7.5), and protein was eluted with 2× Laemmli sample buffer and analyzed by SDS-PAGE and autoradiography as described previously (Ireton et al., 2002). Quantification was performed by densitometry using Image Gage software (Fujifilm Inc.). Arbitrary densitometry units were plotted with GraphPad Prism (GraphPad Software, Inc.) and adjusted for background. Biotinylation of total surface cadherin was performed as described above, but without the pulse-chase labeling.

Constructs

LZRS–mp120–Neo has been described in detail previously (Ireton et al., 2002). The pRS vector was a gift from Reuven Agami (The Netherlands Cancer Institute, Amsterdam, Netherlands). pRS human p120 siRNA and pRS m siRNA were generated according to Brummelkamp et al. (2002). In brief, a 64-bp linker was inserted into pRS using the BamHI and HindIII sites. Oligos for the linker contained p120-specific sense and corresponding antisense sequences, flanking a 6-base hairpin, and were PAGE purified by Integrated DNA Technologies. pEGFP-C1 δ-catenin (Lu et al., 1999) was a gift from Qun Lu (East Carolina University, Greenville, NC). pEGFP-C2 ARVCF C11 (Waibler et al., 2001) was a gift from Anna Starzinski-Powitz (Johann Wolfgang Goethe-Universität, Frankfurt, Germany). pEGFP-C1 (CLONTECH Laboratories, Inc.) was used as a negative control in transfection experiments.

Western blotting

Western blotting procedures were conducted as described by Mariner et al. (2001). In brief, cells were grown to confluence and lysed with either NP-40 or RIPA buffer. Protein concentrations in lysates were obtained by copper reduction/bicinchoninic acid (BCA) assay (Pierce Chemical Co.) according to the manufacturer's instructions. Primary antibodies were used as follows: mAb pp120 (0.1 μg/ml), anti-E-cadherin mAbs C-20820 (1/2,500) and HECD-1 (0.1 μg/ml), anti-β-catenin pAb C-2206 (1/5,000; Sigma-Aldrich), and anti-α-catenin pAb C-2081 (1/5,000; Sigma-Aldrich). Secondary antibodies were peroxidase-conjugated donkey anti–mouse IgG (1/10,000; Jackson ImmunoResearch Laboratories) and mouse anti–rabbit IgG (1/10,000; Jackson ImmunoResearch Laboratories). Anti-tubulin (DM1a; Sigma-Aldrich) and anti-vinculin (hvin-1; Sigma-Aldrich) were used at 1:1,000 and 1:400, respectively.

Inhibitors

Cells were plated at 5×10^5 cells per 60-mm dish for 36 h before treatment with inhibitors. Inhibitors were added to standard growth media at the following concentrations: 33 nM PS341 (Millenium Pharmaceuticals), 3.3 μM lactacystin (Calbiochem), 33 μM chloroquine (Sigma-Aldrich), 5 mM ammonium chloride (Sigma-Aldrich), and 10 nM IETD-CHO (Calbiochem). Cells were treated with inhibitors for 24 h before lysis in NP-40 buffer and analyzed by Western blotting with E-cadherin mAb HECD-1.

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